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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/809,965	03/25/2004	Dave S.B. Hoon	89212.0016	7891

7590 01/08/2007

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EXAMINER	
CHUNDURU, SURYAPRABHA	
ART UNIT	PAPER NUMBER
1637	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	01/08/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Office Action Summary	Application No.	Applicant(s)	
	10/809,965	HOON ET AL.	
	Examiner	Art Unit	
	Suryaprabha Chunduru	1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *09 November 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-31* is/are pending in the application.

4a) Of the above claim(s) *26-31* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-25* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *25 March 2004* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *1/31/06*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

1. Applicant's election of Group I (claims 1-25), in the reply filed on November 03, 2006 is acknowledged. The response neither provides any indication whether the election is with traverse or without traverse nor provided arguments for traversal. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Status

2. Claims 1-25 are considered for examination. Claims 26-31 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected Group.

Information Disclosure Statement

3. The Information Disclosure Statement filed on January 31, 2006 has been entered and considered in part.

Priority

4. This application filed on March 25, 2004 claims benefit of US provisional 60/457,895 filed on March 25, 2003.

Objection to the Specification

5. The Specification is objected because of the following informalities:

(i) This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply the requirements of 37 CFR 1.821 through 1.825.

The instant application recites sequences that are not identified by SEQ ID No. (see at least page 34-35, page -49) recite a nucleic acid sequence / amino acid sequence with more than 10

nucleotides or 4 amino acids, which is not identified by SEQ ID NO.). Further it is noted that the disclosure contains no sequence listing either in the form of a paper copy or in a computer readable form. Appropriate correction is required.

Informalities

6. The following informalities are noted while examining the application:

(i) The instant specification contains a list of references on page 57-60 and on page 70-78. It is suggested that all the references put together be placed at the end of the disclosure.

Claim Rejections - 35 USC § 102

7. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

A. Claims 13-14, 17-25 are rejected under 35 U.S.C. 102(a) as being anticipated by Yang et al. (Clinical Cancer Res., Vol. 8, pp. 2890-2893, 2002).

Yang et al. teach a method of claim 13-14, 17, 20, 23, for detecting cancer comprising (a) providing a sample from a subject (see page 2890, col. 2, paragraph 1 under Materials and Methods section);

(b) detecting one or more DNA markers in the sample, wherein a combination of LOH and hypermethylation are indicative of cancer (see page 2891, col. 1, paragraph 1-2 under Materials and Methods section, page 2890, col. 1, abstract, page 2892, col. 1, paragraph 1, table-3).

With regard to claim 14, 19, 22, 25, Yang et al. teach that said sample is a tissue sample (see page 2890, col. 2, paragraph 1 under Materials and Methods section).

With regard to claim 18, 21, 24, Yang et al. teach that said cancer comprises breast cancer (see page 2890, col. 2, paragraph 1 under Materials and Methods section).

With regard to the claims 20, 23, Yang et al. teach that the detection of DNA markers in various stages of cancer and poor prognosis is indicative of cancer (see page 2892, col. 2, paragraph 1-2 indicating progression of cancer and poor prognosis of cancer associated with said DNA markers). Accordingly Yang et al. anticipates the instant claims.

B. Claims 13-15, 17,19, 20, 22-23, 25 are rejected under 35 U.S.C. 102(b) as being anticipated by Kondo et al. (Hepatology, Vol. 32, pp. 970-979, 2000).

Kondo et al. teach a method of claim 13, 17, 20, 23, for detecting cancer comprising (a) providing a sample from a subject (see page 971, col. 1, paragraph 1 under Materials and Methods section);

(b) detecting one or more DNA markers in the sample, wherein a combination of LOH and hypermethylation are indicative of cancer (see page 971, col. 1, paragraph 4 under Materials and Methods section, page 972, col. 2, paragraphs 1-3, page 970, col. 1, paragraph 1 (abstract), page 973, Fig. 2).

With regard to claim 14, 19, 22, 25, Kondo et al. teach that said sample is a tissue sample (see page 971, col. 1, paragraph 1 under Materials and Methods section).

With regard to claim 15, Kondo et al. teach that said LOH marker includes D10S197 (see page 972, table 2).

With regard to the claims 20, 23, Kondo et al. teach that the detection of DNA markers in various stages of cancer and poor prognosis is indicative of cancer (see page 973, table 3 (different stages of cancer and poor prognosis of cancer). Accordingly Kondo et al. anticipates the instant claims.

C. Claims 1, 7-12 are rejected under 35 U.S.C. 102(b) as being anticipated by Hoon et al. (WO 96/29430).

Hoon et al. teach a method of claim 1, 7, 11, of detecting DNA markers (nucleic acid markers) in a sample comprising (i) providing a cell-free bone marrow sample (bone-marrow aspirate) (see at least page 5, line 28-31, page 96, line 10-23); (ii) detecting one or more DNA markers in the sample (see page 94, line 3-7, page 4, line 23-30).

With regard to claim 7-8, Hoon et al. teach that the method comprises detecting breast and melanoma cancer in a sample (see at least page 94, line 3-7).

With regard to claims 9-12, Hoon et al. teach that the method comprises detecting staging and prognosis of breast and melanoma cancer (see at least page 42, line 1-38, page 43, line 8-31, page 44, line 1-30, page 45, line 1-33). Accordingly Hoon et al. anticipates the instant claims.

Claim Rejections - 35 USC § 103

8. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole

would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

A. Claims 2-3, 5-6 are rejected under 35 U.S.C. 103(a) as being unpatentable over Hoon et al (WO 96/29430) in view of Anker et al. (Cancer and Metastasis reviews, Vol.18, page 65-73, 1999).

Hoon et al. teach a method for detecting DNA markers in a cancer patient's sample as discussed above in 7C, However, Hoon et al. did not specifically teach that the DNA makers as LOH, DNA hypermethylation or DNA mutation.

Anker et al. teach the detection of circulating DNA markers in plasma of cancer patients wherein Anker et al. teach that the DNA markers include LOH microsatellite markers located on chromosome 3p, hypermethylation markers such as gene p16, MGMT and mutation in KRAS gene as indicators of cancer detection and progression (see page 65, abstract, page 67, col. 2, paragraphs 1-2, page col. 1, paragraphs 1-2, col. 2, paragraph 1-2, page 69, col. 1,paragraph 1-2).

It would have been prima facie obvious to a person of ordinary skill in the art at the time the invention was made to modify the method for detecting DNA markers in cancer samples as taught by Hoon et al. with the DNA markers, including LOH, hypermethylation and mutation as taught by Anker et al. for the purpose of developing a sensitive method for detecting a cancer because Anker explicitly taught the use of said markers in cancer patients and the association of said markers in the progression of cancer (see page 65, abstract, page 69, col. 2, paragraph 1, page 71, col. 1, paragraphs 1-2 under conclusion section). An ordinary person skilled in the art would have a reasonable expectation of success that the combination of the method of Hoon et al. and the DNA markers of Anker et al. would result in a sensitive method for detecting cancer

as Anker et al. explicitly taught that the DNA markers play a major role in diagnostic and screening test for cancer and indicators of cancer detection and progression (see page 71, col. 1, paragraphs 1-2 under conclusion section) and such modification of the method is considered as obvious over the cited prior art.

B. Claim 4 is rejected under 35 U.S.C. 103(a) as being unpatentable over Hoon et al (WO 96/29430) in view of Fujiwara et al. (Cancer Res., Vol. 59, pp. 1567-1571, 1999).

Hoon et al. teach a method for detecting DNA markers in a cancer patient's sample as discussed above in 7C, However, Hoon et al. did not specifically teach that the DNA makers as as claimed in the instant claim 4.

Fujiwara et al. teach a method for detecting DNA markers in cell-free plasma samples in melanoma patients, wherein Fujiwara et al. disclose that said method comprises analysis of various microsatellite markers which includes D1S228 at chromosome 1p (see page 1568, col. 1, paragraph 3, col. 2, table 1).

It would have been prima facie obvious to a person of ordinary skill in the art at the time the invention was made to modify the method for detecting DNA markers in cancer samples as taught by Hoon et al. with the DNA markers as taught by Fujiwara et al. for the purpose of developing a sensitive method for detecting a cancer because Fujiwara et al. explicitly taught the use of microsatellite markers on different chromosomes in detecting cancer and the association of said markers in the progression of cancer (see page 1567, col. 1, abstract). An ordinary person skilled in the art would have a reasonable expectation of success that the combination of the method of Hoon et al. and the DNA markers of Fujiwara et al. would result in a sensitive method for detecting cancer as Fujiwara et al. explicitly taught that the DNA markers play a major role in

detecting cancer (see page1567, col. 1, abstract) and such modification of the method is considered as obvious over the cited prior art.

C. Claim 16 is rejected under 35 U.S.C. 103(a) as being unpatentable over Kondo et al. (Hepatology, Vol. 32, pp. 970-979, 2000) in view of Anker et al. (Cancer and Metastasis reviews, Vol.18, page 65-73, 1999).

Kondo et al. teach a method for detecting DNA markers in a cancer patient's sample as discussed above in 7B, However, Kondo et al. did not specifically teach that the DNA hypermethylation makers as claimed in the instant claim 16.

Anker et al. teach the detection of circulating DNA markers in plasma of cancer patients wherein Anker et al. teach that the DNA markers include LOH microsatellite markers located on chromosome 3p, hypermethylation markers such as gene p16, MGMT and mutation in KRAS gene as indicators of cancer detection and progression (see page 65, abstract, page 67, col. 2, paragraphs 1-2; page col. 1, paragraphs 1-2, col. 2, paragraph 1-2, page 69, col. 1,paragraph 1-2).

It would have been prima facie obvious to a person of ordinary skill in the art at the time the invention was made to modify the method for detecting DNA markers in cancer samples as taught by Kondo et al. with the DNA hypermethylation markers as taught by Anker et al. for the purpose of developing a sensitive method for detecting a cancer because Anker explicitly taught the use of said markers in cancer patients and the association of said markers in the progression of cancer (see page 65, abstract, page 69, col. 2, paragraph 1, page 71, col. 1, paragraphs 1-2 under conclusion section). An ordinary person skilled in the art would have a reasonable expectation of success that the combination of the method of Kondo et al. and the DNA markers of Anker et al. would result in a sensitive method for detecting cancer as Anker et al. explicitly

taught that the DNA markers play a major role in diagnostic and screening test for cancer and indicators of cancer detection and progression (see page 71, col. 1, paragraphs 1-2 under conclusion section) and such modification of the method is considered as obvious over the cited prior art.

Conclusion

No claims are allowable.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Suryaprabha Chunduru whose telephone number is 571-272-0783. The examiner can normally be reached on 8.30A.M. - 4.30P.M , Mon - Friday,.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Suryaprabha Chunduru
Primary Examiner
Art Unit 1637

Suryaprabha Chunduru 1/3/07
SURYAPRABHA CHUNDURU
PRIMARY EXAMINER

(Delete the words on this line if you're sending by 1st class mail; leave the hard return) Express Mail Label No. [#]

FORM PTO-1449	Docket Number (Optional) 89212.0016	Application Number 10/809,965
INFORMATION DISCLOSURE CITATION IN AN APPLICATION *(Use several sheets if necessary)*	Applicant Hoon et al.	
	Filing Date March 25, 2004	Group Art Unit 1634

JAN 31 2006 U.S. PATENT & TRADEMARK OFFICE

U.S. PATENT DOCUMENTS

EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
SC	6,465,177	10/2002	Hoon	435	6	

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

SC	FUJIWARA et al. Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients, Cancer Research, Vol. 59, pp. 1567-1571, 1999
SC	ANKER et al., Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients, Cancer and Metastasis Reviews, Vol. 18, pp. 65-73, 1999

EXAMINER /Suryaprabha Chunduru/	DATE CONSIDERED 01/03/2007

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.

Notice of References Cited	Application/Control No. 10/809,965	Applicant(s)/Patent Under Reexamination HOON ET AL.	
	Examiner Suryaprabha Chunduru	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO 9629430 A1	09-1996	World Intellect	HOON et al.	-------
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Fujiwara et al. Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients, Cancer Research, Vol. 59, pp. 1567-1571, 1999.
*	V	Anker et al. Detection of circulating tumor DNA in the blood (plasma/serum) of cancer patients, Cancer and Metastasis Reviews, Vol. 18, pp. 65-73, 1999.
	W	Yang et al. Two-hit inactivation of FHIT by loss of heterozygosity and hypermethylation in breast cancer. Clinical Cancer Research, Vol. 8, pp. 2890-2893, September 2002.
	X	Kondo et al. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis- a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8CpG islands in microdissected specimens from patients with hepatocellular carcinoma, Hepatology, Vol. 32, pp. 970-979, 2000.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Genetic Instability and Aberrant DNA Methylation in Chronic Hepatitis and Cirrhosis—A Comprehensive Study of Loss of Heterozygosity and Microsatellite Instability at 39 Loci and DNA Hypermethylation on 8 CpG Islands in Microdissected Specimens From Patients With Hepatocellular Carcinoma

YUTAKA KONDO,[1,2] YAE KANAI,[1] MICHIIE SAKAMOTO,[1] MASASHI MIZOKAMI,[2] RYUZO UEDA,[2] AND SETSUO HIROHASHI[1]

A study was conducted to examine the significance of genetic instability and aberrant DNA methylation during hepatocarcinogenesis. Genomic DNA was extracted from 196 microdissected specimens of noncancerous liver tissue that showed no marked histologic findings or findings compatible with chronic hepatitis or cirrhosis, and 80 corresponding microdissected specimens of hepatocellular carcinoma (HCC) from 40 patients. Loss of heterozygosity (LOH) and microsatellite instability (MSI) were examined by polymerase chain reaction (PCR) using 39 microsatellite markers, and DNA methylation status on 8 CpG islands was examined by bisulfite-PCR. In noncancerous liver tissues, LOH, MSI, and DNA hypermethylation were found in 15 (38%), 6 (15%), and 33 (83%) of 40 cases, respectively. The incidence of DNA hypermethylation in histologically normal liver was similar to that in chronic hepatitis and cirrhosis, although neither LOH nor MSI was found in histologically normal liver. In cancerous tissues, LOH, MSI, and DNA hypermethylation were found in 39 (98%), 8 (20%), and 40 (100%) of 40 cases, respectively. CpG islands of the *p16* gene and methylated in tumor 1, 2, 12, and 31 clones were frequently methylated in cancerous tissues, although neither the *thrombospondin-1* nor the *human Mut L homologue (hMLH1)* gene was methylated. Absence of silencing of the *hMLH1* gene by DNA hypermethylation is consistent with the low incidence of MSI in HCCs. The results of this study indicate that LOH and aberrant DNA methylation contribute to hepatocarcinogenesis; DNA hypermethylation in particular, which precedes or may even cause LOH, is as an early event during hepatocarcinogenesis. (HEPATOLOGY 2000;32:970-979.)

Hepatocellular carcinoma (HCC) is one of the most frequent human malignancies worldwide. Most of the HCCs that arise in the liver exhibit features compatible with chronic hepatitis and cirrhosis caused by persistent viral infection. In chronic hepatitis or cirrhosis, which are considered to be precancerous conditions, clonal expansion of hepatocytes is initiated.[1,2] Evaluating the genetic events in these conditions leads to a better understanding of multistage hepatocarcinogenesis and may contribute to the choice of effective strategies for preventing the development of HCCs.

DNA methylation of CpG islands that are located near gene promoters affects the transcription of specific genes.[3-7] Aberrant DNA methylation around the promoters of the *E-cadherin* and *p16* genes has been reported in precancerous conditions and HCCs.[7-10] Recently, Toyota et al.[11] described two types of DNA methylation: the methylation of CpG islands in a cancer-specific manner (type C methylation) and age-dependent methylation (type A methylation). The DNA methylation status of type C CpG islands may be a good marker for the alteration of DNA methylation in cancers. CpG islands of the *p16*, *human Mut L homologue1* (*hMLH1*), and *thrombospondin-1* (*THBS-1*) genes and the methylated in tumor (MINT) 1, 2, 12, 25, and 31 clones all exhibit type C methylation.[11,12] Although the gene whose expression was regulated by the MINT clones was not identified, silencing of the *p16*, *THBS-1*, and *hMLH1* genes by DNA hypermethylation may affect the regulation of the cell cycle, angiogenesis, and mismatch repair in human cancers, respectively.[8-10,13-15]

Moreover, aberrant DNA methylation is considered to be associated with chromosomal instability.[16-19] Indeed, Makos et al. reported that all of the renal cancers[20] and neural tumors[21] they detected with loss of heterozygosity (LOH) at the D17S5 locus, where a candidate tumor suppressor gene, *HIC-1*, was identified, also showed DNA hypermethylation at the same locus, and the incidence of DNA hypermethylation exceeded that of LOH. They suggested that DNA hypermethylation precedes or may even cause LOH. With respect to hepatocarcinogenesis, we have reported that regional DNA hypermethylation on chromosome 16q, which is a hot spot for both aberrant DNA methylation and LOH in HCCs, was frequently detected even in precancerous conditions, and pre-

Abbreviations: HCC, hepatocellular carcinoma; *hMLH1*, *human Mut L homologue 1*; *THBS-1*, *thrombospondin-1*; MINT, methylated in tumor; LOH, loss of heterozygosity; MSI, microsatellite instability; HBV, hepatitis B virus; HCV, hepatitis C virus; PCR, polymerase chain reaction; MSP, methylation-specific polymerase chain reaction.

From the [1]Pathology Division, National Cancer Center Research Institute, Tokyo, Japan; and the [2]Second Department of Medicine, Nagoya City University Medical School, Nagoya, Japan.

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doi:10.1053/jhep.2000.19797

ceded LOH at the same locus.[22] In addition, our data on DNA methylation and allelic status at the D17S5 locus in HCC cases[23] supported the suggestion of Makos et al.[20,21] In these analyses, however, conventional Southern blotting was performed using genomic DNA extracted from bulk tissues, and LOH was not detected in noncancerous liver tissues that showed chronic hepatitis or cirrhosis, although aberrant DNA methylation was detected even in precancerous conditions.

Recently, microdissection techniques and methodologies have been developed. LOH in small numbers of microdissected cells from paraffin-embedded tissues has been detected in noncancerous tissues accompanying non–small-cell lung cancer[24] and breast cancer.[25] We recently showed that DNA hypermethylation on chromosome 16q certainly precedes the LOH that occurs there, and can be detected even at the stage of chronic hepatitis or cirrhosis using the microdissection technique.[26] These findings encouraged us to perform a comprehensive study of genetic instability and aberrant DNA methylation on multiple chromosomal loci in microdissected specimens from noncancerous liver tissue obtained from HCC patients. To assess the significance of genetic and epigenetic changes in multistage hepatocarcinogenesis, especially in the early stage, we examined LOH and microsatellite instability (MSI), using 39 microsatellite markers, and DNA methylation status on 8 type C CpG islands in microdissected noncancerous liver tissues that showed no marked histologic findings, findings compatible with chronic hepatitis or cirrhosis, and corresponding cancerous tissues from HCC patients.

MATERIALS AND METHODS

Tissue Samples. Paired samples of noncancerous liver tissue and cancerous tissue were obtained from 40 patients with HCC (cases 1 to 40) who underwent therapeutic surgical resection at the National Cancer Center Hospital, Tokyo, Japan (Table 1). Histologic examination of the noncancerous liver tissues revealed no remarkable findings, or findings compatible with chronic hepatitis or cirrhosis in 5, 20, and 15 cases, respectively. Hepatitis B virus (HBV) surface antigen and anti–hepatitis C virus (HCV) antibody were measured serologically. HBV DNA in liver tissue specimens was also tested for by seminested polymerase chain reaction (PCR) using a pair of primers encompassing the HBV region (positions 72 to 586 based on a sequence from GenBank, accession number M12906), sense: 5′-TACAGGCGGGGTTTTTCTTTG-3′ and antisense: 5′-AAGCCCTGCAACCACTGAA-3′, for first-round PCR and sense: 5′-TACAGGCGGGGTTTTTCTTTG-3′ and antisense: 5′-AAGAGGTTGGTGAGTGATTG-3′ for second-round PCR.

In addition, samples of normal liver tissue were also obtained from 8 patients without HBV or HCV infection who underwent partial hepatectomy for liver metastasis of primary colon cancer.

DNA Preparation. Tissue dissection was performed using an 18-gauge needle and a stereoscopic microscope. Five lobules per case were dissected from noncancerous liver tissues that showed no marked histologic findings, and 4 or 5 pseudolobules or regenerative nodules per case were dissected from noncancerous liver tissues that showed findings compatible with chronic hepatitis or cirrhosis (N1 to N5 for each case, total 196 samples). Two specimens of 1 mm^3 per case were dissected from cancerous tissues (T1 and T2 for each case, total 80 samples). Four lobules per case were dissected from normal liver tissues obtained from the 8 patients with liver metastases of primary colon cancer, and used for comparison. Genomic DNA was extracted and purified from the microdissected samples by the phenol-chloroform method.[27]

Analysis of LOH and MSI. Genomic DNA was amplified by PCR using oligonucleotide primers for 39 microsatellite markers corresponding to previously reported sequences (Table 2).[28] We selected microsatellite markers that we and another group had used previously for detecting LOH and MSI in nonmicrodissected specimens of HCC.[29,30] LOH and MSI were detected as described previously.[30] Briefly, the 5′ ends of the primers were labeled with 6-carboxyfluorescein, and PCR amplifications were performed with 10 ng of genomic DNA. Subsequently, the PCR products were fractionated by electrophoresis (ABI 310 sequencer; Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Data were analyzed using the GeneScan computer program (Applied Biosystems). When 2 amplified bands per locus were detected in at least 1 of the microdissected noncancerous liver tissue specimens, the case was defined as informative for LOH analysis. LOH was recorded when the relative intensity of one allele was reduced by more than 70% in informative cases.[30] MSI was identified by the presence of band shifts or the presence of novel bands in the PCR products. All examinations

TABLE 1. Background of Patients With Hepatocellular Carcinomas

Case	Age	Sex	Tumor Differentiation*	Tumor Size (cm)	Noncancerous† Liver Tissue	Viral‡ Status
1	71	F	M	3.8	LC	C
2	71	M	M	2.5	CH	NBNC
3	54	F	M	6	CH	B
4	77	M	P	5	CH	C
5	65	M	M	5	CH	C
6	58	M	P	5	CH	B
7	66	M	M	11	N	NBNC
8	48	M	M	9	CH	C
9	48	M	W	9.5	N	NBNC
10	66	M	M	4	CH	C
11	66	M	P	11	LC	C
12	20	M	M	3	N	B
13	48	M	P	7.5	LC	B
14	67	F	M	4	LC	C
15	64	M	M	1.3	CH	C
16	61	M	P	13	CH	B
17	74	M	P	1.6	LC	C
18	53	F	M	13	CH	B
19	56	M	M	2	CH	B&C
20	76	M	M	4.5	N	NBNC
21	65	M	M	9	LC	C
22	71	F	M	2	LC	C
23	75	M	M	3	LC	C
24	40	M	M	3	LC	NBNC
25	65	M	M	4.5	LC	C
26	59	M	M	13.8	CH	C
27	72	M	M	3	LC	NBNC
28	68	M	P	2	CH	C
29	66	M	M	3	CH	C
30	66	M	M	5	CH	B
31	54	M	M	5	LC	B
32	72	F	P	8	CH	C
33	77	F	M	3	LC	C
34	69	M	M	6.5	CH	C
35	29	M	M	3	CH	B
36	62	M	M	4	LC	B
37	71	M	W	5	CH	C
38	66	M	M	3.5	CH	C
39	61	F	M	4	LC	C
40	68	M	M	5	N	NBNC

*W, well differentiated hepatocellular carcinoma; M, moderately differentiated hepatocellular carcinoma; P, poorly differentiated hepatocellular carcinoma.

†N, histologically normal; CH, chronic hepatitis; LC, liver cirrhosis.

‡B, HBV DNA positive; C, anti-HCV antibody positive; NBNC, HBV DNA and anti-HCV antibody negative; B&C, both HBV DNA and anti-HCV antibody positive.

TABLE 2. Characteristics of Microsatellite Markers

Marker	Name	Chromosomal Location
1	BAT25	4q12
2	BAT40	1p13.1
3	APC (D5S346)	5q21-22
4	D17S250	17q11.2-12
5	D16S408	16q
6	D16S164	16q21-22.1
7	D16S168	16q21-22.1
8	IFNA	9p22
9	MYCL1	1p32
10	D18S69	18q21
11	D10S197	10qter
12	UT762	21
13	ACTBP2	6q
14	AR	X
15	DRPLA	12
16	D16S409	16q
17	D16S410	16p
18	D17S261	17p12-11.1
19	TP53CA	17p13.1
20	CA21	2p
21	BAT26	2p22-21
22	D2S123	2p16
23	D3S1611	3p
24	BAX	19q13.3-13.4
25	IGF-IIR	6q26-27
26	TGFβ-IIR	3p22
27	hMSH3	5q11.2-13.3
28	hMSH6	2p16-15
29	E2F-4	16q21-22
30	D1S199	1p36
31	D1S235	1q42-43
32	D2S336	2q36-37
33	D4S426	4q35
34	D6S305	6q27
35	D7S493	7p15
36	D8S277	8p23
37	D8S258	8p22
38	D13S171	13q12-13
39	D17S786	17p13

were performed in duplicate in each of 2 separate PCR runs. The results of the 2 experiments were in agreement.

Analysis of Aberrant DNA Methylation. Bisulfite conversion was performed with 1 μg of genomic DNA using the reagents provided in a CpGenome DNA Modification Kit (Intergen, Purchase, NY). Treatment of DNA with bisulfite converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged.[31]

Methylation-specific PCR (MSP) was performed to evaluate DNA methylation status on CpG islands of the *p16* and *hMLH1* genes. MSP was based on the principle that the DNA sequences of methylated and unmethylated genomic regions after bisulfite conversion differ and are distinguishable by sequence-specific PCR primers.[32] For the *p16* and *hMLH1* genes, the bisulfite-modified DNA was amplified using primers provided in a CpG WIZ amplification kit (Intergen) and previously described primers,[15] respectively.

The DNA methylation status of the *THBS-1* gene and MINT 1, 2, 12, 25, and 31 clones was determined by combined bisulfite restriction enzyme analysis.[33] The bisulfite-modified DNA was amplified by PCR using primers designed to amplify methylated and unmethylated genomic regions equally. The PCR product was then digested with restriction enzymes that digested DNA only if the CpG site in their recognition sequence had been methylated before bisulfite treatment and was not converted to TpG. The PCR conditions and primers were as reported previously.[11] The amplified fragments were digested with the restriction enzymes *Taq*I and *Bst*UI, *Taq*I *Taq*I, and *Bst*UI, *Mae*II, *Rsa*I, and *Bst*UI for the *THBS-1* gene and the MINT 1, 2, 12, 25, and 31 clones, respectively.

The reaction products were separated by 2% (MSP) or 3% (combined bisulfite restriction enzyme analysis) agarose gel electrophoresis and stained with ethidium bromide. The signal intensities were evaluated with an FMBIO-2 image analyzer (Takara, Ohtsu, Japan).

Statistical Analysis. The χ^2 test, Fisher's test, Student's *t* test, Spearman's test, and the Kruskal-Wallis test were used for statistical analysis. Differences at $P < .05$ were considered to be significant.

RESULTS

LOH in Precancerous Conditions and HCCs. Examples of the PCR products are shown in Fig. 1. The results of LOH analysis are illustrated in Fig. 2.

In noncancerous liver tissues, LOH for at least one marker was found in 31 (16%) of 196 microdissected samples, and

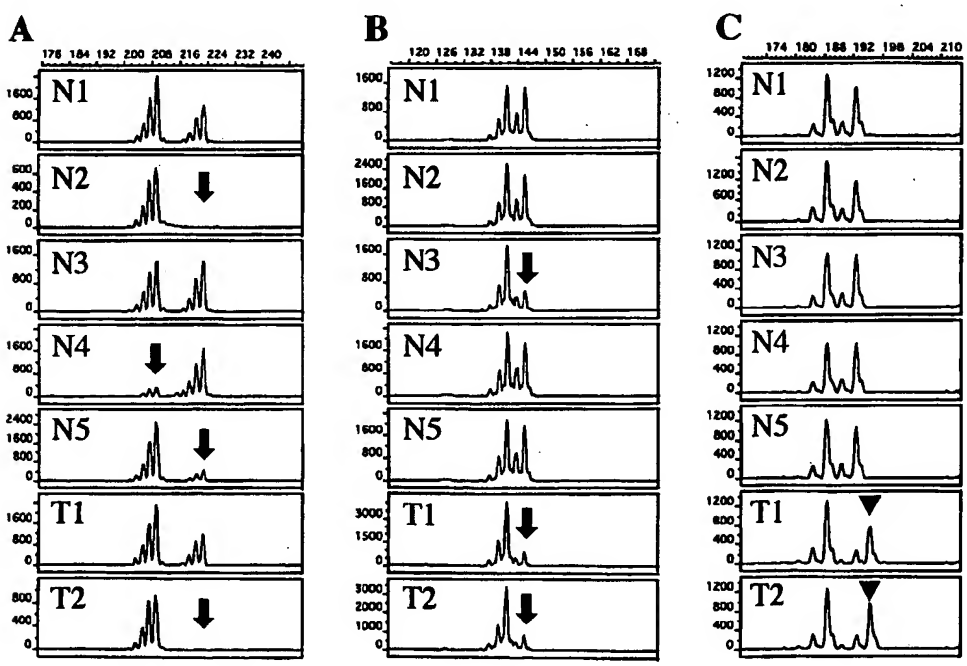

FIG. 1. Examples of results of LOH and MSI analyses in HCC cases. DNA samples obtained from patients 24, 15, and 39 were amplified with markers 22 (A), 37 (B), and 14 (C), respectively. Genotypes derived from noncancerous liver tissue (N1 to N5) and corresponding cancerous tissues (T1 to T2) are shown. Allele sizes (in bp) are indicated on the top horizontal axis. Fluorescence units are on the vertical axis. LOH was identified when the relative intensity of one allele was reduced by more than 70% in an informative case (*arrows*). MSI was identified by the presence of band shifts or the presence of novel bands in the PCR products (*arrowheads*).

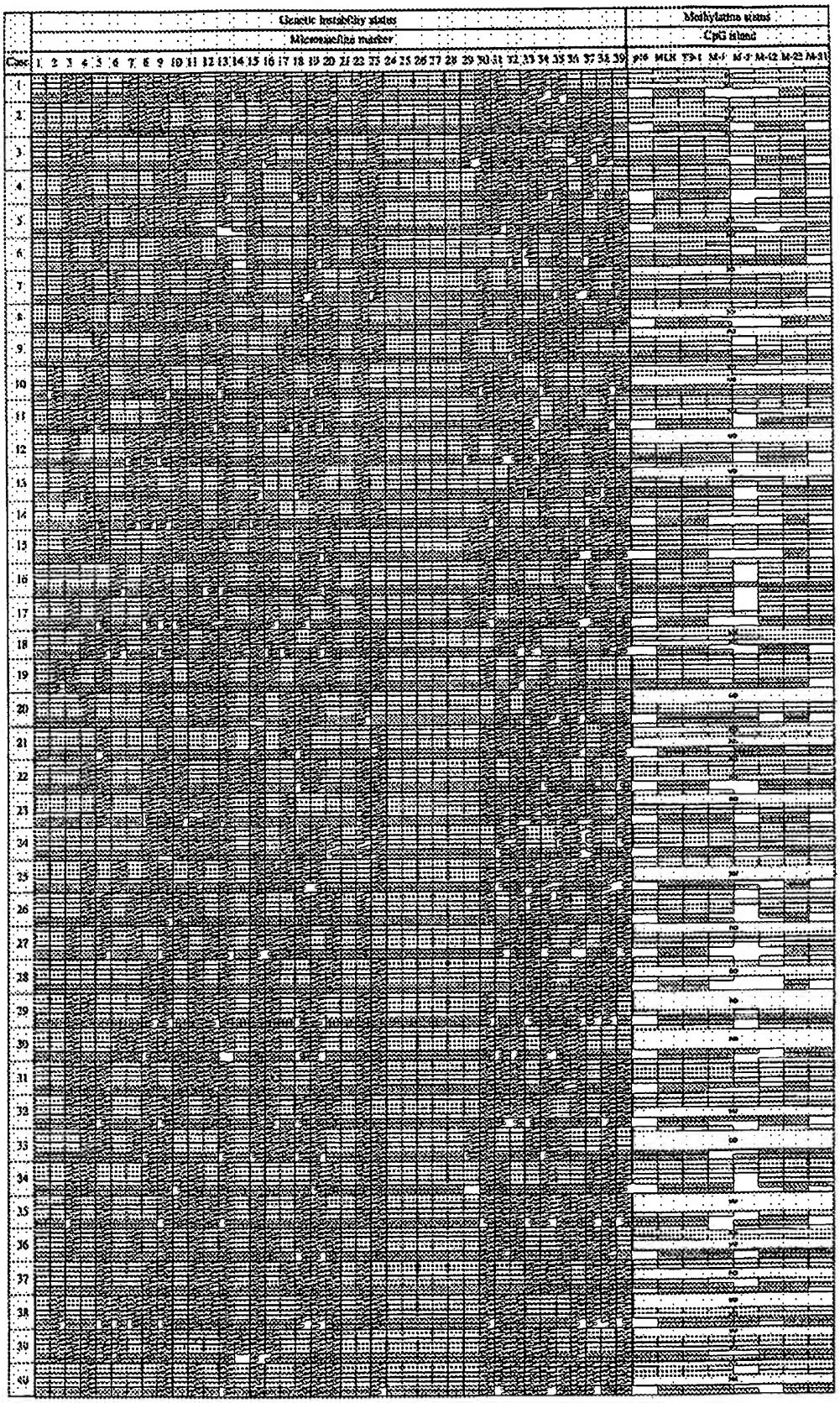

FIG. 2. Genetic instability and DNA methylation status examined in 40 HCC cases. Case numbers are indicated on the vertical column. In each case, *white rows* indicate samples microdissected separately from noncancerous liver tissues and *gray rows* indicate samples microdissected separately from cancerous tissues. Thirty-nine microsatellite markers and 8 CpG islands are indicated on the *top row*. The number of microsatellite markers corresponds to those in Table 2. MLH, TS-1, M-1, -2, -12, -25, and -31 indicate *hMLH-1*, *thrombospondin-1*, MINT 1, MINT 2, MINT 12, MINT 25, and MINT 31, respectively. In the genetic instability status group the symbols indicate the following: *box with oblique line*, informative case for LOH; *box without oblique line*, not informative case for LOH; *black triangle on left upper corner of box*, loss of upper allele; *black triangle on right lower corner of box*, lower allele deletion; *black box*, RER detected. In DNA methylation status group the symbols indicate the following: *black box*, methylated; *white box*, unmethylated; *ND in white blank without separation*, not done.

TABLE 3. Loss of Heterozygosity, Microsatellite Instability, and Aberrant DNA Methylation in Hepatocellular Carcinoma Cases

	Analysis of Genetic Instability			Analysis of DNA Methylation Status	
	Analyzed*	Loss of Heterozygosity† Detected (%)	Microsatellite‡ Instability Detected (%)	Analyzed	DNA Hypermethylation§ Detected (%)
Normal liver tissue¶	32	0 (0)	0 (0)	32	0 (0)
	8	0 (0)	0 (0)	8	0 (0)
Noncancerous liver tissue	196	31 (16)	8 (4)	122	64 (52)
from hepatocellular carcinoma patients	40	15 (38)	6 (15)	40	33 (83)
Histologically normal	25	0 (0)	0 (0)	12	7 (58)
	5	0 (0)	0 (0)	5	4 (80)
Chronic hepatitis	98	20 (20)	2 (2)	64	33 (52)
	20	9 (45)	2 (10)	20	17 (85)
Cirrhosis	73	11 (15)	4 (5)	46	24 (52)
	15	6 (40)	4 (27)	15	12 (80)
Hepatocellular carcinoma	80	75 (94)	12 (15)	80	76 (95)
	40	39 (98)	8 (20)	40	40 (100)

*Upper rank: number of microdissected specimens; lower rank: number of cases.

†Upper rank: number of microdissected specimens in which loss of heterozygosity was detected on at least one marker; lower rank: number of cases in which loss of heterozygosity was detected in at least one microdissected specimen.

‡Upper rank: number of microdissected specimens in which microsatellite instability was detected on at least one marker; lower rank: number of cases in which microsatellite instability was detected in at least one microdissected specimen.

§Upper rank: number of microdissected specimens in which aberrant DNA methylation was detected on at least one CpG island; lower rank: number of cases in which aberrant DNA methylation was detected in at least one microdissected specimen.

¶Normal liver tissue samples were obtained from patients with liver metastasis of primary colon cancer.

LOH in at least 1 microdissected sample was found in 15 (38%) of 40 cases (Table 3). The incidence of LOH detected in noncancerous liver tissue that showed histologic findings compatible with chronic hepatitis (9 of 20 cases, 45%) was similar to that in noncancerous liver tissues that showed histologic findings compatible with cirrhosis (6 of 15 cases, 40%), although LOH was not detected in noncancerous liver tissues that showed no marked histologic findings. Some microdissected pseudolobules and regenerative nodules, such as the samples from cases 1, 2, 3, 8, 23, 24, 25, and 26, showed LOH in multiple loci (Fig. 2). In cancerous tissues, LOH for at least 1 marker was found in 75 (94%) of 80 microdissected samples and LOH for at least 1 microdissected sample was found in 39 (98%) of 40 cases (Table 3).

The incidence of LOH on individual loci is summarized in Table 4. The highest incidence of LOH was observed on 4q (D4S426), 8p (D8S258), and X (AR) in noncancerous liver tissues. In addition to these 3 loci, a high incidence of LOH was also detected on 1p (BAT40), 6q (D6S305), 8p (D8S277), 16q (D16S408 and D16S164), and 17p (D17S261, TP53CA, and D17S786) in cancerous tissues.

Nonspecific allele loss, *i.e.*, absence of an allele in a sample of noncancerous liver tissue that differs from the allele absent in a corresponding HCC, was seen for markers 14, 16, 20, 22, 23, 30, 31, 33, 34, and 38 located on X, 16q, 2p, 2p, 3p, 1p, 1q, 4q, 6q, and 13q, respectively (Fig. 2). Several cases showed LOH on specific markers in noncancerous liver tissue but not in the corresponding HCC. For example, case 1 showed LOH on markers 22 and 31 in noncancerous liver tissue but not in the corresponding HCC (Fig. 2). In 18 (45%) of the 40 cases, both of the microdissected samples obtained from an HCC showed LOH, and each sample lacked the same allele that was lost in the other, whereas heterogeneity of the allelic status *i.e.*, LOH in only one of the microdissected samples, was observed in the other 21 (53%) HCCs (Fig. 2).

There was no significant difference in viral status, incidence of HBV DNA positivity, or anti-HCV antibody positivity between cases with and without LOH in both the noncancerous liver tissues and HCCs.

MSI in Precancerous Conditions and HCCs. Examples of the PCR products are shown in Fig. 1. The results of MSI analysis are illustrated in Fig. 2.

In noncancerous liver tissues, MSI for at least 1 marker was found in 8 (4%) of 196 microdissected samples and MSI in at least 1 microdissected sample was found in 6 (15%) of the 40 cases (Table 3). MSI was not detected in noncancerous liver tissues that showed no marked histologic findings (Table 3). The incidence of MSI in noncancerous liver tissues exhibiting histologic findings that are compatible with cirrhosis (4 of 15 cases, 27%) was higher than that in noncancerous liver tissues exhibiting histologic findings compatible with chronic hepatitis (2 of 20 cases, 10%). In cancerous tissues, MSI for at least one marker was found in 12 (15%) of 80 microdissected samples and MSI in at least 1 microdissected sample was found in 8 (20%) of the 40 cases (Table 3). Considering all loci together, the incidence of MSI was 5% or less in noncancerous liver tissues and 8% or less in cancerous tissues (Table 4). There was no significant difference in viral status between the cases with and without MSI in both the noncancerous liver tissues and HCCs.

Aberrant DNA Methylation in Precancerous Conditions and HCCs. Figure 3 shows examples of the PCR products, and Fig. 2 shows the results of analysis of DNA methylation status.

None of the examined normal liver tissues obtained from the patients with liver metastases from primary colon cancer showed any degree of DNA methylation on any of the examined CpG islands (Table 3). In noncancerous liver tissues from HCC patients, DNA hypermethylation was found on at least 1 CpG island in 64 (52%) of 122 microdissected samples and in at least 1 microdissected sample in 33 (83%) of the 40

TABLE 4. Incidence of Genetic Instability in Each Chromosomal Location

Locus	Microsatellite* Marker	Number of Cases†			
		Loss of Heterozygosity Detected/Informative (%)		Microsatellite Instability Detected (%) (n = 40)	
		Noncancerous Liver Tissue	Hepatocellular Carcinoma	Noncancerous Liver Tissue	Hepatocellular Carcinoma
1p	2	0/5 (0)	3/5 (60)	0 (0)	0 (0)
	9	0/37 (0)	14/37 (38)	0 (0)	1 (3)
	30	1/31 (3)	8/31 (26)	0 (0)	0 (0)
1q	31	5/26 (19)	10/26 (38)	0 (0)	0 (0)
2p	20	2/29 (7)	2/29 (7)	0 (0)	0 (0)
	21	0/0 (0)	0/0 (0)	0 (0)	0 (0)
	22	6/32 (19)	3/32 (9)	0 (0)	0 (0)
	28	0/0 (0)	0/0 (0)	0 (0)	0 (0)
2q	32	2/34 (6)	8/34 (24)	0 (0)	0 (0)
3p	23	1/27 (4)	4/27 (15)	0 (0)	0 (0)
	26	0/0 (0)	0/0 (0)	0 (0)	0 (0)
4q	33	8/28 (29)	15/28 (54)	0 (0)	0 (0)
	1	0/0 (0)	0/0 (0)	0 (0)	0 (0)
4	15	0/37 (0)	8/37 (22)	1 (3)	1 (3)
5q	3	2/22 (9)	2/22 (9)	0 (0)	0 (0)
	27	0/0 (0)	0/0 (0)	0 (0)	0 (0)
6q	13	0/40 (0)	9/40 (23)	0 (0)	2 (5)
	25	0/0 (0)	0/0 (0)	0 (0)	0 (0)
	34	6/32 (19)	14/32 (44)	0 (0)	0 (0)
7p	35	5/29 (17)	6/29 (21)	0 (0)	0 (0)
8p	36	3/27 (11)	12/27 (44)	0 (0)	1 (3)
	37	6/25 (24)	16/25 (64)	1 (3)	0 (0)
9p	8	1/28 (4)	7/28 (25)	0 (0)	0 (0)
10q	22	1/28 (4)	1/28 (4)	1 (3)	0 (0)
13q	38	4/28 (14)	10/28 (36)	0 (0)	0 (0)
16p	17	1/13 (8)	3/13 (23)	0 (0)	0 (0)
16q	5	2/24 (8)	12/24 (50)	0 (0)	0 (0)
	6	1/7 (14)	5/7 (71)	0 (0)	0 (0)
	7	1/14 (7)	3/14 (21)	0 (0)	0 (0)
	16	1/20 (5)	8/20 (40)	0 (0)	0 (0)
	29	0/7 (0)	3/7 (43)	0 (0)	1 (3)
17p	18	2/28 (7)	15/28 (54)	0 (0)	0 (0)
	19	0/36 (0)	17/36 (47)	0 (0)	0 (0)
	39	4/28 (14)	13/28 (46)	0 (0)	0 (0)
17q	4	0/23 (0)	0/23 (0)	0 (0)	0 (0)
18q	10	0/24 (0)	4/24 (17)	0 (0)	0 (0)
19q	24	0/0 (0)	0/0 (0)	0 (0)	0 (0)
21	12	1/21 (5)	7/21 (33)	1 (3)	0 (0)
X	14	2/6 (33)	3/6 (50)	2 (5)	3 (8)

*Microsatellite markers are defined in Table 2.

†Number of cases in which genetic instability was detected in at least one microdissected specimen.

cases (Table 3). The incidence of aberrant DNA methylation did not differ among noncancerous liver tissues that showed no marked histologic findings (4 of 5 cases, 80%), chronic hepatitis (17 of 20 cases, 85%) or cirrhosis (12 of 15 cases, 80%). In cancerous tissues, DNA hypermethylation was found on at least 1 CpG island in 76 (95%) of 80 microdissected samples and in at least 1 microdissected sample in all (100%) cases (Table 3).

The incidence of aberrant DNA methylation in each CpG island is summarized in Table 5. DNA hypermethylation on the *p16* gene and MINT 1, 2, and 12 clones was found even in noncancerous liver tissues. Note that aberrant DNA methylation on MINT 2 was most frequently found in both noncancerous liver tissue and cancerous tissues. In contrast, aberrant DNA methylation on the *THBS-1* and *hMLH1* genes was found in neither noncancerous liver tissue nor HCC.

There was no significant difference in the viral status of either noncancerous liver tissues and HCCs between the cases with and without aberrant DNA methylation.

DISCUSSION

LOH has been reported previously on the 8p, 13q (*Rb*), 17p (*p53*), and 16q loci in regenerative nodules from HCC patients.[34-36] In this study we extended the analysis of LOH to include multiple loci and newly found frequent LOH on 4q (*D4S426*, 4q35) and X (*AR*) even in noncancerous as well as cancerous liver tissues. Inactivation of the genes located on chromosome 4q or X might be involved in the early stage of hepatocarcinogenesis.

The incidence of LOH in noncancerous liver tissues that showed findings compatible with chronic hepatitis (45%) was similar to that for cirrhotic liver tissues (40%). In addition,

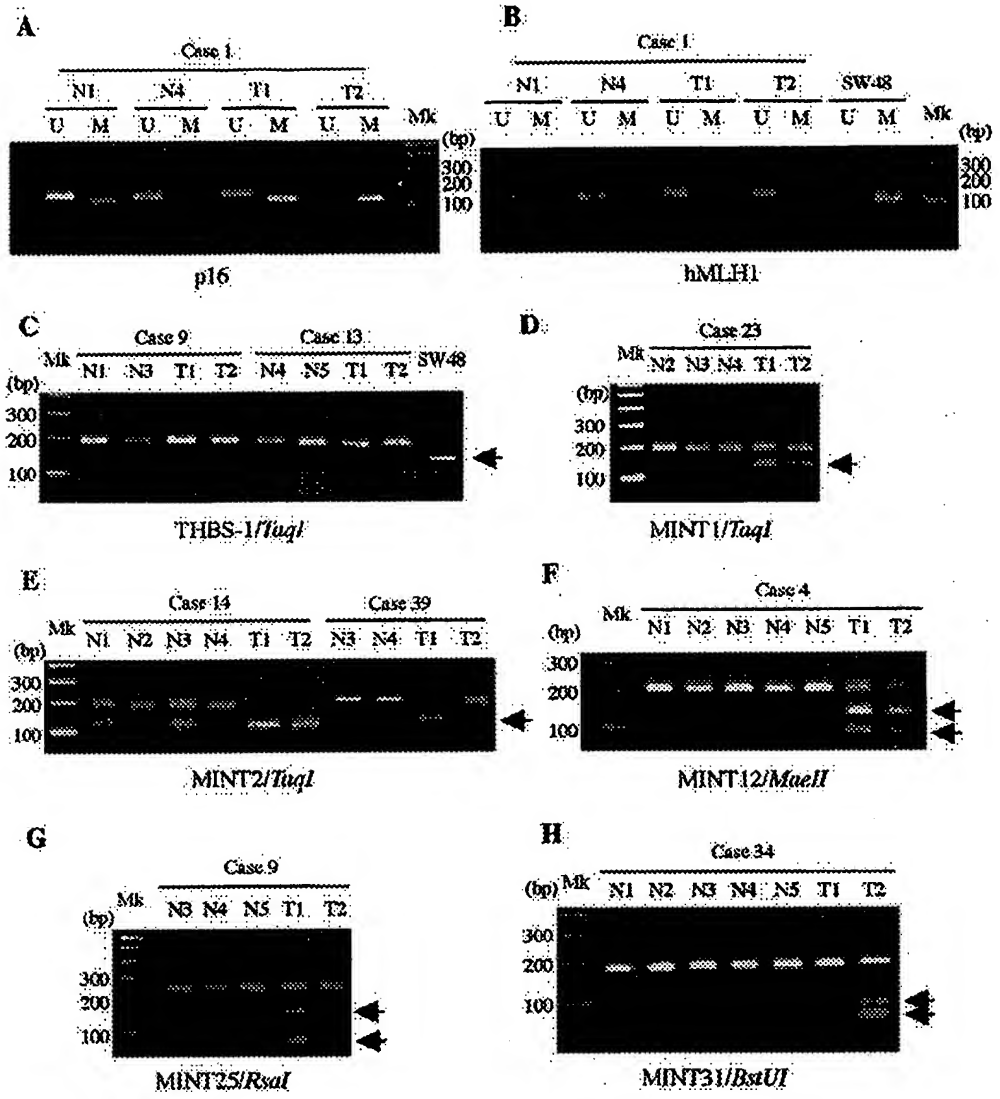

FIG. 3. Examples of results of DNA methylation analysis on multiple CpG islands in HCC cases. DNA methylation status on CpG islands of the *p16* (A) and *hMLH1* (B) genes was evaluated by MSP. In this analysis, a PCR product in the lanes labeled M and U reflects the presence of methylated and unmethylated genes, respectively. DNA methylation status on CpG islands of the *THBS-1* (C) gene and MINT1, 2, 12, 25, and 31 (D, E, F, G, and H, respectively) clones was evaluated by combined bisulfite restriction enzyme analysis. In this type of analysis, only methylated genes are digested by the restriction enzymes, and these are indicated by *arrows*. The restriction enzyme used for digestion is indicated in italics, and case and sample numbers are indicated above each gel. N, noncancerous liver tissue; T, cancerous tissue. SW48 is a colon cancer cell line in which CpG islands of the *hMLH1* and *THBS-1* genes have been reported to be hypermethylated.[24,25] Mk indicates size standard. In some DNA methylation-positive cases, unmethylated alleles were also detected in the same sample. This phenomenon may be attributable, at least in part, to the heterogeneity of DNA methylation status in the sample.

some pseudolobules harbored LOH on multiple loci, suggesting that genetic instability had already accumulated even at the chronic hepatitis stage, in which persistent viral infection and sequential inflammation have occurred.

Recently, in HCC patients, allele-specific loss at the *Rb* (13q)[35] and *p53* loci (17p)[36] was reported in noncancerous liver tissues that showed findings compatible with cirrhosis, suggesting that an unknown mechanism might lead to the preferential loss of one allele at least on some loci. However, nonspecific allele loss on 8p[34] and 16q[26] has also been reported in noncancerous liver tissues from HCC patients showing findings compatible with cirrhosis. In the present study, nonspecific allele loss was seen for 10 markers. Whether or not LOH is allele specific may be determined by the affected chromosomal loci. On chromosomal loci without allele-preferential pressure, either of 2 alleles may be randomly lost among multiple clones of progenitor cells during the precancerous stage. After one of these clones develops into an HCC, some of the other clones continue to lack the allele that differs from the allele that is absent in the corresponding HCC. Moreover, several cases showed LOH on specific markers in noncancerous liver tissues but not in the corresponding HCCs. The reason for this phenomenon may be that even a clone of progenitor cells without LOH on a particular marker can acquire a growth advantage and develop into an HCC.

Allelic status—that is, whether or not LOH was present—was often inconsistent between 2 microdissected specimens from an HCC. Genetic events including allele loss seem to be

TABLE 5. Incidence of Aberrant DNA Methylation in Each CpG Island

CpG Island	Number of Cases (%)*	
	Noncancerous Liver Tissue (n = 40)	Hepatocellular Carcinoma (n = 40)
p16	6 (15)	28 (70)
hMLH1	0 (0)	0 (0)
THBS-1	0 (0)	0 (0)
MINT1	4 (10)	18 (45)
MINT2	29 (73)	34 (85)
MINT12	9 (23)	18 (45)
MINT25	0 (0)	3 (8)
MINT31	0 (0)	26 (65)

*Number of cases in which aberrant DNA methylation was detected in at least one microdissected specimen.

more frequently heterogeneous, even in an HCC, than the current understanding would suggest, based on conventional analysis using nonmicrodissected specimens. This heterogeneity may at least partly explain the molecular mechanisms responsible for malignant progression as a nodule-in-nodule type lesion; a progressed HCC component often emerges within nodules of early HCC which correspond to carcinoma *in situ* or microinvasive carcinoma.[37,38] LOH may occur in a progressed HCC component before it has emerged in the surrounding early HCC component in a nodule-in-nodule type lesion.

With respect to the affected locus in HCCs, an LOH on marker 9 (MYCL1) was frequently found. The L-*myc* gene, which codes a member of the myc oncoprotein family, was amplified and overexpressed in small cell lung cancers.[39] On the other hand, an LOH on the MYCL1 locus has been reported in non–small-cell lung cancers, and the possibility that a tumor susceptibility gene is located in proximity to the MYCL1 locus has been proposed.[40] The high LOH frequency on the MYCL1 locus in HCCs suggests the possible inactivation of the predicted tumor susceptibility gene during hepatocarcinogenesis.

Our recent study of MSI in HCCs using nonmicrodissected specimens showed that MSI was a rare event during hepatocarcinogenesis and might rather be especially associated with HCC progression.[30] In this study we extended our analysis of MSI to include microdissected tissue specimens, and used an increased number of microsatellite markers. The microdissection technique allowed us to detect MSI even in noncancerous liver tissues. The incidence of MSI in cirrhotic liver tissues (27%) was almost 3 times higher than that in noncancerous liver tissues exhibiting findings that are compatible with chronic hepatitis (10%). In addition, the incidence of MSI in cirrhotic liver tissues does not differ from the incidence in HCCs. MSI seems to occur at a later stage than LOH during hepatocarcinogenesis. Taken together with our previous findings that MSI was especially associated with malignant progression, cirrhotic liver tissues with MSI appear to rapidly generate HCCs, which are already at a progressed stage when diagnosed. E2F4 is a transcription factor that influences the progression through the G1-S transition of the cell cycle,[41] and MSI in trinucleotide repeats of the *E2F-4* gene have been documented in gastrointestinal tumors.[42] We detected MSI on marker 29 (E2F-4), although it was only in an HCC (case 34), and the LOH on this marker was frequently found in HCCs. Therefore, the possibility that the inactivation of E2F4 may result in abnormal cell proliferation in HCCs cannot be ruled out. At any rate, none of the patients fulfilled the criterion of microsatellite instability-high, that has been proposed for colorectal cancers.[43] MSI was confirmed to be a rare event during hepatocarcinogenesis, at least in Japanese patients, as previously proposed.[30]

We previously reported DNA hypermethylation around the CpG islands of the *E-cadherin* tumor suppressor gene[7] and at the *D17S5* locus,[23] at which a candidate tumor suppressor gene, *HIC-1*, was identified, in noncancerous liver tissues from HCC cases that showed findings compatible with chronic hepatitis and cirrhosis, even using nonmicrodissected specimens. In this study we extended our analysis of DNA methylation status on 8 type C CpG islands to include microdissected specimens. DNA hypermethylation on the *p16* gene and MINT 1, 2, and 12 clones was frequently found in noncancerous liver tissues. In addition to these CpG islands, MINT 31 was frequently methylated in HCCs. The incidence of DNA hypermethylation on each CpG island was even higher in HCCs than in the corresponding noncancerous liver tissues, thus confirming that aberrant DNA methylation is an early and major event of hepatocarcinogenesis, as we have proposed previously.[7,22,23,44]

In particular, the incidence of DNA hypermethylation on MINT 2 was extremely high in both noncancerous liver tissues (73%) and HCCs (85%) compared with that for previously reported colorectal and gastric cancers.[11,45] Tumor-suppressor protein, which is silenced by DNA hypermethylation and plays a critical role in early hepatocarcinogenesis, may be encoded downstream of MINT 2. DNA methylation was not found on the *hMLH1* or *THBS-1* gene in our cohort, differing from the situation reported previously for colorectal and endometrial cancers and glioblastoma.[11,14,46] During hepatocarcinogenesis, these two genes may not become targets of aberrant DNA methylation. Indeed, the low incidence of MSI in HCCs is compatible with absence of silencing of the *hMLH1* gene by DNA hypermethylation.

Aberrant DNA methylation was found even in noncancerous, microdissected liver tissue samples that showed no marked histologic findings, and may be an earlier event than LOH, which was not found in histologically normal liver from HCC patients. Aberrant methylation of DNA seems to occur even before inflammation has become histologically obvious. This phenomenon might be at least partly attributable to hepatitis viral infection: HBV DNA is integrated in the cellular genome[47] and the integrated viral DNA is known to alter the DNA methylation status in several adjacent cellular genes and DNA segments.[48-50] By contrast, LOH seems to occur solely during the persistent inflammation and regeneration of hepatocytes.

We have reported that aberrant DNA methylation preceded LOH at the same locus on 16q, which was the hot spot for both aberrant DNA methylation and LOH in HCCs, in chronic hepatitis, and cirrhosis.[26] The markers we used in this study, 20, 21, and 22 (CA21, BAT26, and D2S123, respectively), were located on 2p adjacent to the MINT 2 clone. On 2p, DNA hypermethylation and LOH tended to occur together or not at all (55%), and the incidence of DNA hypermethylation alone was also high (41%) in microdissected specimens of noncancerous liver tissue. However, the incidence of LOH alone (4%) was significantly lower than in the former two cases ($P < .01$), reflecting our previous findings for 16q.[26] There are two possible explanations for this: (1) common or different causes facilitate both aberrant DNA methylation and LOH at certain loci, and DNA methylation status changes immediately prior to allelic status; or (2) aberrant DNA methylation itself predisposes the same locus to allelic loss. Although our data do not indicate which of these two possibilities is the case, *in vitro* and *in vivo* evidence suggesting that aberrant DNA methylation affects chromosomal instability—probably through chromatin configuration changes—is accumulating.[16-19] One can consider the possibility that aberrant DNA methylation may even cause LOH on at least some loci during carcinogenesis.

Understanding the significance of aberrant DNA methylation in the precancerous stage may present a new strategy—correction of DNA methylation status—for preventing HCC

in hepatitis virus carriers suffering from chronic hepatitis or cirrhosis.

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Two-Hit Inactivation of *FHIT* by Loss of Heterozygosity and Hypermethylation in Breast Cancer[1]

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ABSTRACT

Purpose: **The *FHIT* gene, which spans the FRA3B fragile site at chromosome 3p14.2, is a candidate tumor suppressor gene in breast carcinomas. In this study, we would like to delineate more precisely its role in breast tumorigenesis.**

Experimental Design: **To confirm the tumorigenic role of *FHIT*, 46 sporadic invasive ductal carcinomas of the breast were tested for the "two hits" required to inactivate this gene. Microsatellite loss of heterozygosity (LOH) was considered as the first hit. To examine the possibility that hypermethylation serves as the second hit for *FHIT* inactivation, methylation of 5′-CpG islands of *FHIT* was analyzed by methylation-specific PCR.**

Results: **LOH was detected in 8 of 40 informative tumors, and hypermethylation was observed in 22 of 46 (48%) cases. Aberrant FHIT protein expression was found in 31 of 46 (67%) cases examined. All seven tumors showing both LOH and hypermethylation showed complete loss of Fhit protein expression. In addition, a significant positive association was found between the existence of LOH and 5′-CpG island hypermethylation ($P = 0.04$), which was consistent with the two-hit model.**

Conclusions: **To our knowledge, this study provides the first evidence that biallelic inactivation of *FHIT* by LOH and hypermethylation leads to the complete inactivation of *FHIT* gene in patients with breast cancer. Silencing of the *FHIT* gene by promoter hypermethylation occurs in primary breast carcinomas, especially those with LOH. These findings support a role for this tumor suppressor gene in sporadic breast tumorigenesis.**

INTRODUCTION

Multiple genetic abnormalities characterize invasive breast cancers (1–3), including LOH[3] at chromosomal sites that harbor known or putative tumor suppressor genes. In breast cancer, LOH frequently occurs at several 3p regions and includes 3p14.2, 3p21, and 3p24 (4–7). The tumor suppressor gene *FHIT*, located at chromosome 3p14.2, is more than 1 Mb in size and encodes a 1.1-kb cDNA with 10 small exons; exon 5 is the first protein coding exon, and it is flanked in intron 4 and intron 5 by the most common fragile site in the human genome, FRA3B (6). The *FHIT* gene belongs to the histidine triad (8) superfamily and encodes a cytoplasmic M_r 16,800 protein with diadenosine triphosphate hydrolase activity. The conserved histidines are required for full enzymatic activity (8). The gene is frequently inactivated in many tumor types, including those of breast, cervix, esophagus, digestive tract, and lung (6, 9–17). However, it remains uncertain which mechanisms, apart from LOH, are behind the above-mentioned loss of function of Fhit in Japanese breast tumors.

Point mutations of *FHIT* are very infrequent events (18). An alternative mechanism to intragenic mutations for the inactivation of tumor suppressor genes is promoter hypermethylation (19). In particular, hypermethylation of normally unmethylated CpG islands located in the promoter regions of many tumor suppressor and DNA repair genes, such as *RARβ2*, *E-cadherin*, and *BRCA1*, is associated with loss of gene expression in cancer cell lines and primary tumors (20–22). A previous report (23) suggests that aberrant methylation of *FHIT* could occur in breast carcinoma. In this study, we examined the role of LOH and hypermethylation for *FHIT* inactivation in Japanese breast carcinomas.

MATERIALS AND METHODS

Tissue Samples. A total of 46 study subjects were selected from 61 consecutive breast carcinoma patients in our breast cancer research project. All specimens underwent histological examination by two pathologists to confirm diagnosis of adenocarcinoma through evaluation of >90% of tumor cells constituting these samples. Paired tumor and peripheral blood samples were collected from the Affiliated Kihoku Hospital of Wakayama Medical University, Japan. Tumor samples were

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[3] The abbreviation used is: LOH, loss of heterozygosity.

Table 1 Relationship between *FHIT* locus alteration and Fhit protein expression

	Fhit expression		
	Negative	Positive	*P*
ROH[a]	20	12	0.08
LOH	7	1	

[a] ROH, retention of heterozygosity.

snap-frozen at –70°C immediately after resection. Peripheral blood samples were immediately subjected to isolation of DNA.

Microsatellite Analysis of LOH. DNA extractions were performed using the QIAamp Tissue Kit (Qiagen) according to the manufacturer's protocols. Analysis of PCR-based LOH was performed by using three microsatellite markers flanking chromosome 3p14.2: (*a*) *D3S1300*; (*b*) *D3S1481*; and (*c*) *D3S1234*. All primer sequences and their locations were obtained from human genetic linkage maps. PCR was carried out in reaction volumes of 50 μl containing 100 ng of genomic DNA as template, 1× PCR buffer, 200 μM deoxynucleotide triphosphate mix, 300 nM forward primer, 300 nM reverse primer, and 2.5 units of Taq DNA polymerase. Each microsatellite marker was amplified from paired normal and tumor DNA samples by PCR under the following reaction conditions: 94°C for 2 min for one cycle; followed by 35 cycles of 94°C for 1 min, 52–60°C for 30 s, and 72°C for 45 s; with a final incubation at 72°C for 5 min. Ten-μl aliquots of the PCR products were then loaded onto 6% denaturing polyacrylamide gels and separated by electrophoresis at 350 V for 3–6 h. Gels were stained using the PlusOne DNA Silver Staining Kit in a GeneStain Automated Gel Stainer (Pharmacia Biotech AB). Two observers analyzed the staining results visually and recorded allele imbalance when there was clear reduction in the intensity of one allele amplified from tumor DNA samples.

Methylation-specific PCR. Approximately 1.0 μg of each DNA sample was bisulfite modified by using a commercial kit (CpGenome DNA modification kit; Oncor Inc., Gaithersburg, MD) according to the manufacturer's instructions. Treatment of genomic DNA with sodium bisulfite converts unmethylated but not methylated cytosines to uracil, which is then converted to thymidine during the subsequent PCR step, producing sequences between methylated and unmethylated DNA. Bisulfite-modified DNA was PCR-amplified by using the primer pairs as described previously (23).

Immunohistochemical Staining for Fhit Protein. Paraffin-embedded, 4-μm-thick sections from all 46 tumors were stained for the Fhit protein as described previously (12). Briefly, paraffin-embedded sections on silane-coated slides were dewaxed with xylene and rehydrated through a graded alcohol series. Then, endogenous peroxidase activity was blocked in absolute methanol solution containing 1% hydrogen peroxide for 35 min, and the slides were washed in 10 mM PBS (pH 7.4). For antigen retrieval, they were immersed in 1 mM citrate-phosphate buffer and microwaved at 100°C for 15 min. After the buffer had cooled, normal horse serum was reacted with the slides for 15 min to eliminate nonspecific immunostaining. The slides were reacted with primary polyclonal rabbit IgG antibody

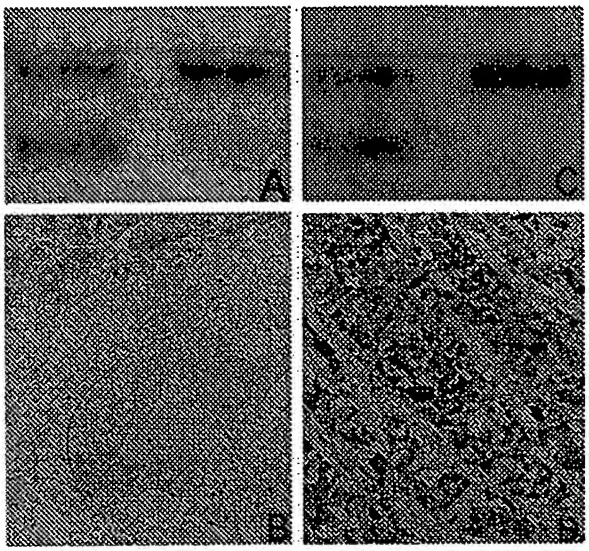

Fig. 1 LOH analysis by using *D3S1300* (*A* and *C*) and immunohistochemical analysis of Fhit protein (*B* and *D*). Case 1: *A*, allelic loss demonstrated by denaturing gel electrophoresis; *B*, immunohistochemistry for Fhit showed completely unreactive tumor cell nests with a residual normal duct structure with strong Fhit immunoreactivity. Case 2: *C*, allelic loss demonstrated by denaturing gel electrophoresis; *D*, diffuse cytoplasmic staining for Fhit demonstrated by immunohistochemical analysis.

to Fhit (ZR44; Zymed Laboratories, Inc., San Francisco, CA) at a dilution of 1:200, overnight at 4°C in a humidified chamber. After reaction with a mouse biotinylated secondary antibody, antigen-antibody reactions were visualized using a streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit; DAKO, Los Angeles, CA) with diaminobenzidine as the chromogen. All slides were counterstained with hematoxylin. Staining without antibody was performed as a negative control. For immunohistochemical evaluation of Fhit, cytoplasmic labeling of tumor cells was classified as follows: negative, no staining or positive staining present in <10% of tumor cells; and positive, positive staining present in ≥10% of tumor cells.

Statistical Analysis. Statistical analysis was performed using StatView 5.0 statistical analysis software (Abacus Concepts, Berkeley, CA). Standard χ^2 test or, when appropriate, Fisher's exact test was used to analyze the association between two categorical variables. All *P*s were two-tailed, and the 0.05 level was considered statistically significant.

RESULTS

Allelic Loss and Aberrant Fhit Protein Expression in Breast Carcinoma. Forty of the 46 patients were informative for at least one locus, and the overall LOH frequency at 3p14.2 involving at least one marker was 20% (8 of 40). By immunohistochemical analysis, Fhit-positive staining was detected in 15 cases (33%). Aberrant Fhit expression was observed in 31 cases, and complete loss of Fhit was identified in 22 cases. As shown in Table 1, 20 of the 32 (63%) tumors with retention at 3p14 showed reduced Fhit expression compared with 7 of the 8 (88%)

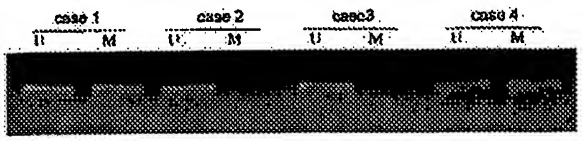

Fig. 2 Methylation analysis of *FHIT* in breast carcinomas. *U*, unmethylated; *M*, methylated. Lanes that do not show a band represent samples that are not methylated.

Table 2 Relationship between *FHIT* methylation and Fhit protein expression

	Fhit expression		
	Negative	Positive	*P*
Methylation			
Negative	16	8	>0.9
Positive	15	7	

tumors with LOH at this region. There was a trend toward a higher Fhit aberration in tumors with LOH ($P = 0.08$), although the difference was not statistically significant (Fig. 1).

Frequent *FHIT* Hypermethylation in Breast Carcinoma. Fhit methylation was found in 22 of 46 (48%) breast carcinomas. Inconsistent with a previous report (23), the unmethylated form of *FHIT* was found in 100% of the cases (Fig. 2). It was assumed that the unmethylated form was the result of stromal or normal tissue contamination. No significant correlation between *FHIT* methylation and Fhit expression was observed (Table 2). To determine whether *FHIT* hypermethylation is associated with the frequent loss of chromosomal material at one allele of *FHIT* observed in breast carcinoma, we examined the relationship between hypermethylation and LOH. Of the 40 informative cases for microsatellite analysis, 20 were demonstrated to have hypermethylation. Aberrant methylation was found in seven of eight (88%) tumors with LOH. As shown in Table 3, a significant correlation with hypermethylation and LOH was observed ($P = 0.04$).

Two-Hit Inactivation of *FHIT* in Breast Cancer. In many cancers, biallelic inactivation of suppressor genes is the result of mutation of one allele followed by deletion of the remaining allele. This two-step process can be observed as LOH involving polymorphic markers linked to suppressor gene loci (24). When LOH at the *FHIT* locus was analyzed in the breast carcinomas, 8 of the 40 informative cases showed LOH. Interestingly, of the eight tumor samples that exhibited LOH, the seven cases with methylation showed complete loss of Fhit protein. Therefore, biallelic inactivation of the *FHIT* suppressor gene may result from epigenetic modification of one allele followed by gene deletion of the remaining allele.

DISCUSSION

The *FHIT* gene and its protein product have been the focus of recent debate with regard to their potential role in tumorigenesis (25). A tumor suppressor role for Fhit has been postulated based on the ability of Fhit to eliminate or reduce the tumorigenicity of tumor cells in nude and knockout mice (26, 27). Clinicopathologically, aberrant Fhit expression has been associated with pathogenesis and prognosis of various tumors (6, 9–17). However, the mechanism of *FHIT* suppression remains largely unknown.

Table 3 Relationship between methylation and *FHIT* locus loss

	FHIT locus		
	ROH[a]	LOH	*P*
Methylation			
Negative	19	1	0.04
Positive	13	7	

[a] ROH, retention of heterozygosity.

The *FHIT* gene is located at chromosome 3p14.2, and LOH of this region has been detected in 25–45% of breast carcinomas (4, 10, 28, 29). Compared with these previous reports, the 20% LOH rate detected in our present study was not high. This may be a result of our sample selection bias. It is thought that LOH alone cannot completely suppress Fhit expression because many genes can be expressed monoallelicly (7, 30, 31). Two hits are required to inactivate tumor suppressor genes (32), and hypermethylation should also be considered as one of the hits (33, 34). Silencing by abnormal promoter methylation of Rb, VHL, MLH1, p16, and BRCA1 associated with inactivation of the other allele by a "classical hit," such as intragenic mutation or LOH, is a relatively common finding in human cancer. Our findings fit this model, demonstrating the strong association between *FHIT* hypermethylation and the existence of LOH at the *FHIT* locus. Previous studies have demonstrated that mutations are very infrequent in *FHIT* (18, 28, 35, 36). These data suggest that, in breast carcinomas, one allele is lost by deletion, and the other is inactivated by aberrant methylation, with both events leading simultaneously to the biallelic inactivation and complete lack of function of the *FHIT* gene. However, some tumors with reduced Fhit expression had no LOH or methylation, and other mechanisms such as splicing abnormality should be considered (10).

Notably, the rate of hypermethylation at *FHIT* is higher than the percentage of LOH at the *FHIT* locus, which suggests that *FHIT* hypermethylation is a more common event in breast carcinoma. Biallelic inactivation of the *FHIT* gene could result from epigenetic inactivation of both parental alleles and could be reversed by exposure to demethylating agents (23). It is tempting to speculate that demethylating agents might have a role in cancer prevention for individuals who are at risk for cancer or for individuals in whom *FHIT* promoter methylation is detected as an early neoplastic change. Moreover, knowledge of the *FHIT* methylation state in primary breast cancers may be useful to identify tumors that are more likely to respond to *FHIT*-demethylating therapy.

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(54) Title: DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

(57) Abstract

A method for the diagnosis of melanoma or breast cancer is provided. In particular, the method provides for the detection of nucleic acids corresponding to multiple melanoma or breast cancer specific markers using template-dependent amplification processes. In one embodiment, the markers used are tyrosinase, MUC18, p97, MAGE-3, β-HCG, MAGE-1 and GalNAc. The methods using these combinations of markers are more sensitive in the detection of tumor cells in patients as compared to single marker assays.

DESCRIPTION

DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

BACKGROUND OF THE INVENTION

Some of the work described in this application was supported by grant number PO1 CA1038 from the National Cancer Institute.

1. Field of the Invention

The present invention relates generally to the field of cancer diagnostic techniques. In particular, the invention relates to the detection of genetic markers indicative of melanoma or breast cancer cells. In one example, detection of multiple markers is achieved by polymerase chain reaction assay.

2. Description of the Related Art

Cancers are one of the leading causes of disease, being responsible for 526,000 deaths in the United States each year (Boring *et al.*, 1993). For example, breast cancer is the most common form of malignant disease among women in Western countries and, in the United States, is the most common cause of death among women between 40 and 55 years of age (Forrest, 1990). The incidence of breast cancer is increasing, especially in older women, but the cause of this increase is unknown. Malignant melanoma is another form of cancer whose incidence is increasing at a frightening rate, at least sixfold in the United States since 1945, and is the single most deadly of all skin diseases (Fitzpatrick, 1986).

One of the most devastating aspects of cancer is the propensity of cells from malignant neoplasms to disseminate from their primary site to distant organs and develop into metastases. Despite advances in surgical treatment of primary neoplasms and aggressive therapies, most cancer patients die as a result of metastatic disease. Animal tests indicate that about 0.01% of circulating cancer cells from solid tumors establish successful metastatic colonies (Fidler, 1993).

Thus, the detection of occult cancer cells in circulation is important in assessing the level of tumor progression and metastasis. Because subclinical metastasis can remain dormant for many years, monitoring of patients' blood for circulating tumor cells may prove advantageous in detecting tumor progression before metastasis to other organs occurs. Assessment of circulating tumor cells also would provide a rapid monitoring system to determine if a specific therapy is effective.

For example, recognition of metastases in tumor-draining lymph nodes (TDLN) now has been shown critical for patient management. It is known that between 25-30 per cent of breast cancer patients with node negative, localized disease will relapse within five years after operative intervention (Henderson *et al.*, 1989). Accurate axillary staging of TDLN in detection of metastases has been an important factor for selecting patients for adjuvant therapy (NIH, 1992; Giuliano, *et al.*, 1995; Giuliano, *et al.*, 1994). Several retrospective studies on breast cancer TDLN demonstrated that analysis of multiple sections of nodes shown to be tumor negative were found to have occult metastases (Bettelheim, *et al.*, 1990; Chen *et al.*, 1991; Neville *et al.*, 1991). The identification of nodes with occult metastases were shown to significantly correlate to

poorer prognosis (Bettelheim *et al.*, 1990; Neville *et al.*, 1991).

Previous tumor diagnostic techniques have focused on the detection of tumor associated antigens or on molecules released by tumor cells (Smart, 1990; Moertel *et al.*, 1993; Stamey *et al.*, 1989). At best, these assays only detect tumors with no indication of metastatic potential or tumor progression. In addition, such assays measure a single antigen whose release is often proportional to the size of the tumor and they cannot account for heterogeneity of individual markers in tumor lesions, both within individual patients or among patient groups.

The recent development of the PCR assay (Mullis and Faloona, 1987; Erlich, 1989) for detection of occult metastatic tumor cells in blood using specific markers has provided a new approach to assess tumor progression (Smith *et al.*, 1991; Naito *et al.*, 1991). In one study, circulating melanoma cells in blood were detected by PCR analysis using the tyrosinase gene marker (Smith *et al.*, 1991). Seven melanoma patients with metastatic disease were analyzed, but only four were positive. Other studies using PCR have been used to detect circulating tumor cells in melanoma, as well as in breast, prostate and neuroblastoma cancer patients (Smith *et al.*, 1991; Datta *et al.*, 1994; Moreno *et al.*, 1992; Naito *et al.*, 1991). These studies, employing a single marker, were limited by their ability to discriminate cancer cells from normal cells also carrying the marker, thus reducing specificity and reliability. In addition, tumor heterogeneity has caused sensitivity problems where a single, specific marker has been employed.

As indicated above, tumors are notoriously heterogeneous, particularly in advanced stages of tumor

progression (Morton *et al.*, 1993; Fidler and Hart, 1982; Nowell, 1982; Elder *et al.*, 1989; Bystryn *et al.*, 1985). Although tumor cells within a primary tumor or metastasis all may express the same marker gene, the level of specific mRNA expression can vary considerably (Elder *et al.*, 1989). It is, therefore, necessary to develop a detection system that can cope with such heterogeneous targets.

Thus, despite the identification of melanoma and breast cancer markers, these markers cannot individually detect tumor cells in a highly specific and sensitive manner. This is due to the wide phenotypic diversity found in tumor cells at any one time and during disease differentiation. There remains a need to develop a more sophisticated approach, that can accommodate such a biological heterogeneous situation in order to sensitively and specifically detect metastasis and diagnosis disease stage.

3. Summary of the Invention

The present invention seeks to overcome these and other drawbacks inherent in the prior art by providing sensitive and accurate methods for the detection of melanoma or breast cancer cells in a biological sample. The methods provide for the detection of melanoma or breast cancer cells in a biological sample by amplifying at least two nucleic acids from the sample, the nucleic acids being markers for melanoma or breast cancer cells.

Claim 1

The present invention comprises the following steps. A nucleic acid is extracted from a biological sample. The nucleic acid is contacted with a first primer pair that hybridizes to a first melanoma or breast cancer marker nucleic acid. The primers are extended by polymerase to produce an amplification product. This

process is repeated a sufficient number of times to permit detection of the amplification product. Finally, all steps are repeated with the same biological sample and a second primer pair that hybridizes to another melanoma or breast cancer marker nucleic acid.

In preferred embodiments, the method may further comprise preparing at least two pairs of primers complementary to regions of melanoma or breast cancer marker nucleic acids. In another embodiment, the method may further comprise of preparing primer pairs for at least three, four, five, six or even seven melanoma or breast cancer markers.

In preferred embodiments of the invention, the markers amplified and detected are selected from the group comprising tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG. The preferred method of amplification is by reverse transcription and polymerase chain reaction (PCR). In one embodiment of the invention the PCR further comprises nested PCR.

In one embodiment, the nucleic acid is RNA. Preferably, the RNA extracted from a biological sample is total cellular RNA. In a preferred embodiment, the total cellular RNA is converted to DNA prior to amplification.

In certain embodiments of the invention, the biological sample is a body tissue or body fluid. In preferred embodiments, the body tissue is bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy. Other embodiments include samples where the body fluid is peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or

urine. In a preferred embodiment, the biological sample is of human origin.

In preferred embodiments of the invention, the method includes separation of the amplification product by gel electrophoresis. In other embodiments, the method of separation is by chromatographic techniques. In a preferred embodiment of the invention, hybridization with a labeled probe permits identification of the amplification product following separation.

In further embodiments, the present invention encompasses a kit for use in detecting melanoma or breast cancer cells in a biological sample comprising, pairs of primers for amplifying nucleic acids corresponding to the marker genes, and containers for each of these primers. In preferred embodiments, the kit further comprises enzymes and reagents for the preparation of cDNA's and amplification thereof. In yet more preferred embodiments, the kit further comprises enzymes and reagents for radiochemical or chromophoric labeling of nucleic acids.

4. Detailed Description of the Preferred Embodiments

The present invention pertains to a sensitive, multimarker assay to detect occult melanoma or breast cancer cells in the blood of patients with or without clinical evidence of disease. This assay is designed to overcome limitations in existing technologies with respect to both sensitivity and specificity.

In its most general form, the instant invention comprises a method for identification of melanoma or breast cancer cells in a biological sample by amplifying and detecting nucleic acids corresponding to melanoma or breast cancer cell markers. The biological sample can be

any tissue or fluid in which melanoma or breast cancer cells might be present. Preferred embodiments include bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy. Other embodiments include samples where the body fluid is peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

Nucleic acid used as template for amplification is isolated from cells contained in the biological sample according to standard methodologies. (Sambrook *et al.*, 1989) The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary cDNA. In a preferred embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to genes corresponding to specific markers are contacted with the isolated nucleic acid under conditions that permit selective hybridization. Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via

a system using electrical or thermal impulse signals (Affymax technology, Bellus, 1994).

The foregoing process is conducted at least twice on a given sample using at least two different primer pairs specific for two different specific markers. Following detection, one may compare the results seen in a given patient with a statistically significant reference group of normal patients and melanoma or breast cancer patients. In this way, it is possible to correlate the number and kind of markers with various clinical states.

(i) Melanoma-Specific or Breast Cancer-Specific Markers

While the present invention exemplifies several markers, any marker that is correlated with the presence or absence of melanomas or breast cancer may be used. A marker, as used herein, is any proteinaceous molecule (or corresponding gene) whose production or lack of production is characteristic of a melanoma or breast cancer cell. Depending on the particular set of markers employed in a given analysis, the statistical analysis will vary. For example, where a particular combination of markers is highly specific for melanomas or breast cancer, the statistical significance of a positive result will be high. It may be, however, that such specificity is achieved at the cost of sensitivity, *i.e.*, a negative result may occur even in the presence of melanoma or breast cancer. By the same token, a different combination may be very sensitive, *i.e.*, few false negatives, but has a lower specificity.

As new markers are identified, different combinations may be developed that show optimal function with different ethnic groups or sex, different geographic distributions, different stages of disease, different

degrees of specificity or different degrees of sensitivity. Marker combinations may also be developed, which are particularly sensitive to the effect of therapeutic regimens on disease progression. Patients may be monitored after surgery, hyperthermia, immunotherapy, cytokine therapy, gene therapy, radiotherapy or chemotherapy, to determine if a specific therapy is effective.

One particularly useful combination of markers is tyrosinase and p97. Human tyrosinase is an essential enzyme which regulates the production of melanin (Nordlund *et al.*, 1989; Hoon *et al.*, 1993), a group of brown or black pigments in the skin and eyes of humans. More specifically, tyrosinase catalyzes the conversion of tyrosine to Dopa and of Dopa to dopaquinone. p97, also known as melanotransferrin, is a cell surface sialoglycoprotein that bears some sequence homology to transferrin (Brown *et al.*, 1981; Rose *et al.*, 1986). Like transferrin, p97 binds iron, thereby being implicated in iron metabolism.

There are many other markers that may be used in combination with these, and other, markers. For example, β-human chorionic gonadotropin (β-HCG). β-HCG is produced by trophoblastic cells of placenta of pregnant woman and is essential for maintenance of pregnancy at the early stages (Pierce *et al.*, 1981; Talmadge *et al.*, 1984). β-HCG is known to be produced by trophoblastic or germ cell origin tumors, such as choriocarcinoma or testicular carcinoma cells (Madersbacher *et al.*, 1994; Cole *et al.*, 1983). Also ectopic expression of β-HCG has been detected by a number of different immunoassays in various tumors of non-gonadal such as breast, lung, gastric, colon, and pancreas, etc. (McManus *et al.*, 1976; Yoshimura *et al.*, 1994; Yamaguchi *et al.*, 1989; Marcillac *et al.*, 1992; Alfthan *et al.*, 1992). Although the

function of β-HCG production in these tumors is still unknown, the atavistic expression of β-HCG by cancer cells and not by normal cells of non-gonadal origin suggests it may be a potentially good marker in the detection of melanoma and breast cancer (Tormey *et al.*, 1977; Tormey *et al.*, 1975).

Another exemplary example of a marker is glycosyltransferase β-1, 4-N-acetylgalacto-saminyltransferase (GalNAc). GalNAc catalyzes the transfer of N-acetylgalactosamine by β1,4 linkage onto both gangliosides GM3 and GD3 to generate GM2 and GD2, respectively (Nagata, *et al.*, 1992; Furukawa *et al.*, 1993). It also catalyzes the transfer of N-acetylgalactosamine to other carbohydrate molecules such as mucins. Gangliosides are glycosphingolipids containing sialic acids which play an important role in cell differentiation, adhesion and malignant transformation. In melanoma, gangliosides GM2 and GD2 expression, are often enhanced to very high levels and associated with tumor progression including metastatic tumors (Hoon *et al.*, 1989; Ando *et al.*, 1987; Carubia *et al.*, 1984; Tsuchida *et al.*, 1987a). Gangliosides are also highly expressed in breast cancer cells. The gangliosides GM2 and GD2 are immunogenic in humans and can be used as a target for specific immunotherapy such as human monoclonal antibodies or cancer vaccines (Tsuchida *et al.*, 1987b; Irie, 1985).

GalNAc mRNA may be used as a marker of GM2 and GD2 expression and consequently a marker of either melanoma or breast cancer cells. GalNAc is generally not expressed in normal lymphocytes, epithelial cells, melanocytes, connective tisue or lymph node cells. If detected, it is in very low levels.

Other markers contemplated by the present invention include cytolytic T lymphocyte (CTL) targets. MAGE-3 is a marker identified in melanoma cells and breast carcinoma. MAGE-3 is expressed in many melanomas as well as other tumors and is a (CTL) target (Gaugler *et al.*, 1994). MAGE-1 and MAGE-2 are other members of the MAGE gene family. MAGE-1 gene sequence shows 73% identity with MAGE-3 and expresses an antigen also recognized by CTL (Gaugler *et al.*, 1994). MART-1 is another potential CTL target (Robbins *et al.*, 1994) and may also be included in the present invention.

MUC18 is another marker that is useful in the identification of melanoma cells (Lehmann *et al.*, 1989; Lehmann *et al.*, 1987). MUC18 is a cell surface glycoprotein that is a member of the immunoglobulin superfamily and possesses sequence homology to neural cell adhesion molecules (NCAM). Other mucin family members include MUC1, MUC2, MUC3 and MUC4. These were found to be expressed at a high level in certain tumor cell lines (Hollingsworth *et al.*, 1994) and may also be used as markers in the present invention.

Other members of the immunoglobulin superfamily of adhesions molecules associated with the development of melanoma metastasis (Denton *et al.*, 1992) may be utilized in the present invention. Preferred examples include intercellular adhesion molecule-1 (ICAM-1), NCAM, VCAM-1, and ELAM. Another preferred embodiment of the invention, includes cell adhesion molecules associated with other metastatic diseases, such as carcinoembryonic antigen (CEA) and DCC (deleted in colorectal cancer) (Johnson, 1991).

Other breast or skin cancer associated proteins and their corresponding nucleic acids may also be utilized in the present invention. Preferred examples include

melanoma antigen gp75 (Vijayasardahi *et al.*, 1990), human cytokeratin 8 (HKer 8) (Pittman *et al.*, 1993), high molecular weight melanoma antigen (Natali *et al.*, 1987) and Keratin 19 (K19) (Datta *et al.*, 1994). This list is not intended to be exhaustive, but merely exemplary, for the type and number of potential markers which may be used in the present invention.

Other proteins and their corresponding nucleic acids related to the melanin synthesis pathway may be used as markers, such as tyrosinase related protein 1 and 2 and members of the pMel 17 gene family (Kwon *et al.*, 1993).

Preferred embodiments of the invention involve many different combinations of markers for the detection of melanoma breast cancer cells. Any marker that is indicative of neoplasia in breast cells or melanocytes may be included in this invention. However, preferred embodiments include combinations of tyrosinase, MAGE-3, MUC18, p97, β-HCG, GalNAc and MAGE-1. Table 1, as disclosed herein, represent partially useful combinations of markers which may be employed for the detection of melanoma or breast cancer cells.

Table 1. Preferred Multiple Marker Combinations

Table 1A. Combinations of Six or Seven Multiple Markers.

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	+	+	+
+	+	+	+	+	+	-
+	+	+	+	+	-	+
+	+	+	+	-	+	+
+	+	+	-	+	+	+
+	+	-	+	+	+	+
+	-	+	+	+	+	+
-	+	+	+	+	+	+

Table 1B. Combinations of Five Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	+	-	-
+	+	+	+	-	-	+
+	+	+	-	-	+	+
+	+	-	-	+	+	+
+	-	-	+	+	+	+
-	-	+	+	+	+	+
-	+	+	+	+	+	-
+	+	+	+	-	+	-
+	+	+	-	+	-	+
+	+	-	+	-	+	+
+	-	+	-	+	+	+
-	+	-	+	+	+	+
+	-	+	+	+	+	-
-	+	+	+	+	-	+
+	+	+	-	+	+	-
+	+	-	+	+	-	+
+	-	+	+	-	+	+
-	+	+	-	+	+	+
+	+	-	+	+	+	-
+	-	+	+	+	-	+
-	+	+	+	-	+	+

Table 1C. Combinations of Four Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	-	-	-
+	+	+	-	-	-	+
+	+	-	-	-	+	+
+	-	-	-	+	+	+
-	-	-	+	+	+	+
-	-	+	+	+	+	-
-	+	+	+	+	-	-
+	+	+	-	-	+	-
+	+	-	-	+	-	+
+	-	-	+	-	+	+
-	-	+	-	+	+	+
-	+	-	+	+	+	-
+	-	+	+	+	-	-
-	+	+	+	-	-	+
+	+	+	-	+	-	-
+	+	-	+	-	-	+
+	-	+	-	-	+	+
-	+	-	-	+	+	+
+	-	-	+	+	+	-
-	-	+	+	+	-	-
-	+	+	+	-	+	-
+	+	-	-	+	+	-
+	-	-	+	+	-	+
-	-	+	+	-	+	+
-	+	+	-	+	+	-
+	+	-	+	+	-	-
+	-	+	+	-	-	+
-	-	+	-	-	+	+

Table 1D. Combinations of Three Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
-	-	-	-	+	+	+
-	-	-	+	+	+	-
-	-	+	+	+	-	-
-	+	+	+	-	-	-
+	+	+	-	-	-	-
+	+	-	-	-	-	+
+	-	-	-	-	+	+
-	-	-	+	+	-	+
-	-	+	+	-	+	-
-	+	+	-	+	-	-
+	+	-	+	-	-	-
+	-	+	-	-	-	+
-	+	-	-	-	+	+
+	-	-	-	+	+	-
-	-	-	+	-	+	+
-	-	+	-	+	+	-
-	+	-	+	+	-	-
+	-	+	+	-	-	-
-	+	+	-	-	-	+
+	+	-	-	-	+	+
+	-	-	-	+	-	+
-	-	+	+	-	-	+
-	+	+	-	-	+	-
+	+	-	-	+	-	-
+	-	-	+	-	-	+
-	-	+	-	-	+	+
-	+	-	-	+	+	-
+	+	-	+	+	-	-

Table 1E. Combinations of Two Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
-	-	-	-	-	+	+
-	-	-	-	+	+	-
-	-	-	+	+	-	-
-	-	+	+	-	-	-
-	+	+	-	-	-	-
+	+	-	-	-	-	-
+	-	-	-	-	-	+
-	-	-	-	+	-	+
-	-	-	+	-	+	-
-	-	+	-	+	-	-
-	+	-	+	-	-	-
+	-	+	-	-	-	-
-	+	-	-	-	-	+
+	-	-	-	-	+	-
-	-	-	+	-	-	+
-	-	+	-	-	+	-
-	+	-	-	+	-	-
+	-	-	+	-	-	-
-	-	+	-	-	-	+
-	+	-	-	-	+	-
+	-	-	-	+	-	-

+ markers included in the combination; - markers not included.

(ii) Primers

The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be

provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

In most cases, it will be preferable to synthesize desired oligonucleotides. Suitable primers can be synthesized using commercial synthesizers, such as those supplied by Applied Biosystems (Foster City, CA) using methods well known to those of ordinary skill in the art. Where double-stranded primers are desired, synthesis of complementary primers is performed separately and the primers mixed under conditions permitting their hybridization.

Selection of primers is based on a variety of different factors, depending on the method of amplification and the specific marker involved. For example, the choice of primer will determine the specificity of the amplification reaction. The primer needs to be sufficiently long to specifically hybridize to the marker nucleic acid and allow synthesis of amplification products in the presence of the polymerization agent and under appropriate temperature conditions. Shorter primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the marker nucleic acid and may be more susceptible to non-specific hybridization and amplification.

Primer sequences do not need to correspond exactly to the specific marker sequence. Non-complementary nucleotide fragments may be attached to the 5' end of the primer with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary bases can be interspersed into the primer, provided that the primer sequence has sufficient complementarily, in particular at the 3' end, with the

template for annealing to occur and allow synthesis of a complementary DNA strand.

In preferred embodiments, primers may be designed to hybridize to specific regions of the marker nucleic acid sequence. For example, GC rich regions are favored as they form stronger hybridization complexes than AT rich regions. In another example, primers are designed, solely, to hybridize to a pair of exon sequences, with at least one intron in between. This allows for the activity of a marker gene to be detected as opposed to its presence by minimizing background amplification of the genomic sequences and readily distinguishes the target amplification by size.

Primers also may be designed to amplify a particular segment of marker nucleic acid that encodes restriction sites. A restriction site in the final amplification product would enable digestion at that particular site by the relevant restriction enzyme to produce two products of a specific size. Any restriction enzyme may be utilized in this aspect. This added refinement to the amplification process may be necessary when amplifying a marker nucleic acid sequence with close sequence similarity to other nucleic acids. Alternatively, it may be used as an added confirmation of the specificity of the amplification product.

(iii) Template Dependent Amplification Methods

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990, each of which is incorporated herein

by reference in its entirety. Briefly, in PCR, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated. Preferably a reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternatively, preferred methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641 filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the marker sequence, each pair will bind to opposite complementary strands of the marker such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the marker and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent No. 4,883,750 describes a method similar to LCR for binding probe pairs to a marker sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA which has a region complementary to that of a marker is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of marker molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention. Walker *et al.*, 1992, incorporated herein by reference in its entirety.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR) involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Marker specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having a 3' and 5' sequences of non-specific DNA and middle sequence of specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe identified as distinctive products which are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in British Patent Application No. 2,202,328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR like, template and enzyme dependent synthesis. The primers may be modified by labelling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the marker sequence, the probe binds and is cleaved catalytically. After cleavage, the marker sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the marker sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR. Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has marker specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second marker specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by a polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are

reverse transcribed into double-stranded DNA, and transcribed once against with a polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate marker specific sequences.

Davey *et al.*, EPO No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a first template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a second template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting as a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme

based on the hybridization of a promoter/primer sequence to a marker single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR." Frohman, M.A., 1990 and Ohara *et al.*, 1989, each incorporated herein by reference in their entirety.

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, 1989, incorporated herein by reference in its entirety.

(iv) Separation Methods

Following amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification occurred. In a preferred embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989. In a preferred embodiment, the gel is a 2% agarose gel.

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

(v) Identification Methods

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

Alternatively, separation may be unnecessary. These methods may be collectively termed Sequencing By Hybridization or SBH (Cantor *et al.*, 1992; Drmanac & Crkvenjakov, U.S. Patent No. 5,202,231). Development of certain of these methods has given rise to new solid support type sequencing tools known as sequencing chips. The utility of SBH in general is evidenced by the fact that U.S. Patents have been granted on this technology.

SBH can be conducted in two basic ways, often referred to as Format 1 and Format 2 (Cantor *et al.*, 1992). In Format 1, oligonucleotides of unknown sequence, generally of about 100-1000 nucleotides in length, are arrayed on a solid support or filter so that the unknown samples themselves are immobilized (Strezoska *et al.*, 1991; Drmanac & Crkvenjakov, U.S. Patent No. 5,202,231). Replicas of the array are then interrogated by hybridization with sets of labeled probes of about 6 to 8 residues in length.

In Format 2, a sequencing chip is formed from an array of oligonucleotides with known sequences of about 6 to 8 residues in length (Southern, WO 89/10977; Khrapko *et al.*, 1991; Southern *et al.*, 1992). The nucleic acids of unknown sequence are then labeled and allowed to

hybridize to the immobilized oligos. In another embodiment, hybridization may be detected by electrical or thermal impulse signals (Affymax Technology, Bellus, 1994).

In a preferred method, however, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, where the other member of the binding pair carries a detectable moiety.

In a particularly preferred embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to

carrying out methods according to the present invention.

(vi) Clinical Stages of Malignant Melanoma

Cancers are staged according to a well-defined, elaborate progressive scale, developed by the American Joint Committee on Cancer.

Malignant melanomas can arise in any skin area that contains melanocytes, but body moles, also called pigmented nevi, are particularly vulnerable. Although some moles, especially those on the face and torso, originate in pigment cells, they sometimes contain little pigment and are light in color. All moles are initially benign tumors of varying shape, but it is significant to note that about 20 to 30 percent of all melanomas begin in the pigment cells of moles.

Caught early, melanoma is very often curable. On the other hand, melanomas that are not detected until they have invaded even a few millimeters of the deeper layers of skin have a much poorer prognosis. The five-year survival rate varies considerably depending on stage level. For Stage I and Stage II melanoma, the five-year survival rate is over 80%. However, for Stage IV the survival rate is less that 20% (AJCC, .

A simplified summary of the scale, developed by the American Joint Committee for the Staging of melanoma is presented in Table 2.

Table 2: Staging of Melanoma

Stage I: Primary site, small tumor Negative lymph nodes No detectable metastases
Stage II: Invasion beyound primary site Lymph nodes negative may have one positive No detectable distant metastases
Stage III: Tumors at regional skin or subcutaneous sites, primarily located to lymph nodes.
Stage IV: Tumor of any size Lymph nodes either positive or negative Distant metastases to multiple sites

Metastasis to a distal organ may or may not result in secondary metastasis to other organs. Since subclinical metastasis can remain dormant for many years, monitoring of a patient's blood for circulating tumor cells may be helpful in detecting tumor progression before clinically evident metastases to other organs are detected.

(vii) Clinical Stages of Breast Cancer

Many factors appear to influence the chances of surviving breast cancer. Early detection and treatment are the most important. The overall five-year survival rate is about 75 percent for white women and about 63 percent for black women. This rises to nearly 90 percent for women with Stage I or II cancer that is treated while the cancer is confined to the breast (Scanlon and Strax, 1986).

A simplified summary of the scale, developed by the American Joint Committee for the Staging of Breast Cancer in 1982, is presented in Table 3.

Table 3: Staging of Breast Cancers

Stage I: Small tumor (less than 2 cm or .78 inches) Negative lymph nodes No detectable metastases
Stage II: Tumor greater than 2 cm but less than 5 cm Lymph nodes negative or Tumor less than 5 cm across Lymph nodes positive No detectable distant metastases
Stage III: Large tumor (greater than 5 cm) or Tumor of any size with invasion of skin or chest wall or "grave signs" or Associated with positive lymph nodes in the collarbone area but No detectable distant metastases
Stage IV: Tumor of any size Lymph nodes either positive or negative Distant metastases

(viii) Kit Components

All the basic essential materials and reagents required for detecting melanoma or breast cancer cells in a biological sample, may be assembled together in a kit. This will generally comprise of the preselected primers for two, or more, particular specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Tag, etc.), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification.

Such kits will generally comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair. Preferred pairs of primers for amplifying nucleic acids correspond to the genes for tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG.

polymerase (5 U/μl). The volume of the mixture was brought up to 100 μl. The PCR cycling was performed as for the first reaction except the annealing temperature was 55°C. The preparation of PCR mixture for the temperature cycler was carried out in a designated PCR room in a specified laminar flow hood.

The PCR amplification product was detected by electrophoresis on a 2% agarose gel (GIBCO BRL, Grand Island, N.Y.) and visualized by ethidium bromide staining under ultra violet light. An ϕX174RF DNA/*Hae* III fragment DNA ladder (BRL) was used as a size reference marker for all assays.

B. RESULTS

The screening process involved examining 10 established melanoma cell lines (10^6 cells/line) and 39 normal PBL (10^7 cells/blood draw) as controls. In Table 4, the expression of these markers is shown for melanoma cells and PBL. A positive reaction was considered as a visible specific PCR amplification product by gel electrophoresis stained with ethidium bromide.

Table 4

PCR analysis of melanoma marker genes

Marker gene	Melanoma cell lines	PBL
Tyrosinase	9/10	0/39
p97	10/10	0/39
MAGE-3	8/10	0/39
MUC18	10/10	2/39

RNA was extracted from melanoma and PBL and assessed for expression of individual markers by PCR. Data presented as positive cell lines or PBL over total number of specimens assessed. Positive PCR refers to PCR amplification product assessed by gel electrophoresis.

All four markers were transcribed in all the melanoma lines, except for MAGE-3. A melanoma cell line expressing all four markers would produce cDNA PCR products of size; 284 base pairs (bp), 286 bp, 423 bp and 437 bp (tyrosinase, p97, MAGE-3 and MUC18 respectively) as observed after electrophoresis through an agarose gel with ethidium bromide staining and compared with DNA size markers. In one melanoma cell line, tyrosinase expression by PCR was negative; however, when nested PCR for tyrosinase was performed tyrosinase gene expression was detected. There was no detection of melanoma markers in PBL from 39 normal donors, except two donors which were positive for MUC18 gene transcription. These individuals were tested multiple times from separate blood draws and always remained positive for MUC18. This indicated they were not false positive results due to PCR contamination or contamination from normal tissue during blood drawing.

In all assays, MUC18 nested primer PCR was performed; this procedure increased the sensitivity to allow verification and amplification of weak bands produced by PCR with only MUC18 primers. Melanoma cell lines and PBL were tested at least twice to verify specificity. Respective controls in each assay included samples with positive RNA for the gene being assessed, PCR reagents and primers without RNA, human tumor cell lines which were negative for individual gene expression, and β-actin gene expression.

MAGE-1, a gene family member of MAGE-3, also was tested and found to be transcribed in less than 50% of the melanoma cell lines. It was decided not to use this marker for melanoma, since MAGE-3 is more highly expressed in melanomas and MAGE-3 is usually found when MAGE-1 is expressed (Gaugler *et al.*, 1994). Expression

of both genes were not detected in PBL from normal volunteer donors.

EXAMPLE II

Sensitivity of Multiple Melanoma Markers

A. MATERIALS AND METHODS

RNA was isolated and quantitated from melanoma cell lines positive for individual markers. Specific marker PCR analysis was then carried out on serial diluted RNA as described in Example I.

(i) Southern blot analysis

After electrophoresis of PCR amplification products, agarose gels were transferred overnight onto nitrocellulose membrane (Schleicher & Schull, Keene, N.H.) with 20X SSC buffer as previously described. The cDNA was then UV-crosslinked onto the membrane and hybridized overnight with a digoxigenin labelled probe (Morisaki *et al.*, 1992). After hybridization, the membrane was washed in 2X SSC, 0.1% SDS for 10 min. at room temperature and then in 0.1X SSC, 0.1% SDS for 30 min. at 68°C to remove nonspecific binding (Sambrook *et al.*, 1989). Specific binding was detected using anti-digoxigenin, alkaline phosphatase-conjugated antibody as described by the manufactor (Genius Kit; Boehringer Mannheim, Indianapolis, IN). Tyrosinase probes were either prepared, full-length from PCR cDNA products using the outermost PCR oligonucleotide primers, or 2K bp probes were *Eco* R1 digested from plasmids containing the tyrosinase gene sequence (Kwon *et al.*, 1987). All other probes used in Southern blotting, were prepared from PCR cDNA products using the outermost oligonucleotide primers.

Gel electrophoresis and Southern blotting also was performed automatically using the Automated Electrophoresis System, National Genetics, Inc. and U.S. Patent No. 5,279,721. See above.

B. RESULTS

In general, all markers could be detected at picogram levels of RNA by visual examination of gel-electrophoresed PCR amplification products stained with ethidium bromide. RNA from melanoma cell lines were diluted in a series from 1 to 10^{-9} μg and assessed for markers tyrosinase, p97, MUC18, and MAGE-3. Sensitivity varied for individual lines with different levels of gene expression. In general, mRNA for p97, MUC18, and MAGE-3 was detected around 10-100 *pg* by PCR. Tyrosinase mRNA could be detected at 10-100 *fg* by PCR.

Specificity of the amplification products was demonstrated by Southern blotting with respective specific probes (tyrosinase, p97). Sensitivity of the PCR assay could be enhanced 10- to 100-fold using PCR followed by probe blotting. Nested PCR for tyrosinase enhanced detection levels 10-100 fold above PCR for tyrosinase. However, nested PCR for MUC18 enhanced results about 10-fold compared to standard PCR for MUC18.

EXAMPLE III

Detection of Melanoma Cells Mixed With PBL *in vitro*

A model system mimicking circulating melanoma cells in blood was developed. In this assay, system 10^7 normal PBL were mixed with serial dilutions of melanoma cells (10^6 to 1 cell) and assessed by PCR for individual gene markers. The PCR amplification products were then assessed by ethidium bromide staining of gels and by Southern blot analysis. RT-PCR amplification was also

performed on RNA extracted from 10^7 PBL and 10^1 melanoma cells, as controls. Southern blot analysis performed for tyrosinase verified the specificity of the PCR amplification product and demonstrated enhanced sensitivity. Materials and methods were as described in Examples I and II.

Gel electrophoresis or nested primers analysis demonstrated that melanoma cells could be detected at about 1 cell in 10^7 PBL for tyrosinase, p97 and MUC18. PBL controls were negative for individual markers in both gel staining and Southern blot analysis and in both standard and nested PCR. Specific dilutions of melanoma cells, were also analysed, in 50 million PBL and demonstrated that about 1-5 melanoma cells could be detected in 50 million PBL with nested primer tyrosinase PCR followed by probing with tyrosinase cDNA.

To demonstrate the sensitivity and reproducibility of detecting 1 melanoma cell in 10 million PBL, a Poisson distribution analysis was carried out. In 8 of 11 samples, a positive PCR amplification product developed by tyrosinase PCR was detected by gel electrophoresis. The level of detection was enhanced >90% when tyrosinase nested PCR primers or Southern blot analysis with tyrosinase probe was performed.

EXAMPLE IV

Assessment of Circulating Melanoma Cells in Patients Blood

A. MATERIALS AND METHODS

(i) Patients

All melanoma patient with complete documented physical and medical histories were accrued from JWCI. Melanoma patients studied were AJCC (American Joint Committee on Cancer) stage I, II, III and IV. Patients assessed were NED (no evidence of clinical disease), AWD (alive with clinical disease) or EXP (expired during follow-up). The accrual and study of patients was carried out in a double-blind fashion. The patients' disease status was not known to the individual running the PCR assay nor the analyzer of the PCR data. Clinical disease status was documented at the time of blood drawing and again at 8-15 month follow-up period. PCR data results were not known to individuals recording the patient status during the follow-up period.

Fifteen ml of blood was obtained from patients and collected in sodium citrate tubes. All blood was drawn in the John Wayne Cancer Clinic using the same procedure. Blood was drawn after written consent was obtained from the patient. The protocol for the study was approved by the Saint John's Hospital and John Wayne Cancer Institute Human Subjects Committee. Tubes were centrifuged for 20 min at 2000 x g. The buffy coat was carefully removed and diluted in double distilled water. The cells were washed by centrifugation for 5 min. All other materials and methods were performed as described in Example Iand II.

(ii) Protocol

Materials and methods were as described in Examples I and II. PBL from melanoma patients were examined using an optimized PCR assay detection system. The protocol was as follows: PCR assays were performed to detect transcripts of tyrosinase, p97, MAGE-3, and MUC18. All melanoma patients were subjected to all four tests. If the sample was negative for tyrosinase or MUC18, nested PCR was performed with respective primers. If the PBL specimen was negative in the PCR assay for tyrosinase nested primers and p97 markers, then Southern blot analysis would be performed with respective probes. PBLs negative for all the markers and tests were considered as true negatives.

Initially, PBL isolated by Ficoll-hypaque gradient centrifugation were compared to buffy coat isolated PBL. In the analysis, buffy coat isolated cells appeared better for the detection of circulating melanoma cells in blood by PCR.

B. RESULTS

A summary of the analysis of blood specimens by PCR using multiple markers, as assessed by ethidium bromide stained gels, is shown in Table 5. The greatest number of positive patients was observed with MUC18 (73%), with tyrosinase (59%), p97 (54%) and MAGE-3 (10%) identifying few. Analysis with nested primers of tyrosinase versus tyrosinase primers significantly increased the number of positive patients from 2 to 57. Further analysis of p97-negative and tyrosinase-negative patients with respective specific probes significantly increased the number of positive patients. The most significant increase was observed by Southern blotting with the p97 probe. Six

Table 6

PCR positive patients correlation to Disease status patient status

AJCC Stage	AWD & EXP	NED
I	NP	1/4
II	NP	16/18
III	5/6	23/26
IV	46/48	17/18
Total positive	51/54 (94%)	57/66 (86%)

Values represent patients PCR positive (1 or more markers) over total patients evaluated. NP refers to no patients. AWD & EXP refer to patients AWD and those who were AWD during the blood draw and expired (EXP) during the follow-up period.

The detection of PCR markers was correlated with the Breslow thickness and Clark level of the primary melanoma, after it had been surgically removed. The latter two factors play a role in determining the patients prognosis (Breslow, 1970; Morton *et al.*, 1993). Breslow thickness has been shown to correlate very well with disease progression. Breslow thickness was divided into subgroups of 0.75 mm or less, >0.75 mm to 1.49 mm, ≥1.5 mm to 3.0 mm and >3.0 mm. However, there was no significant correlation of Breslow thickness and detection of PCR markers. Although the majority of the patients were either Clark's level 3 or 4, no significant pattern was observed for Clark's level and number of positive PCR markers. Neither the number of tumor-positive regional lymph nodes nor the sites of distal metastases significantly correlated with the number of positive PCR markers.

The lack of correlation between primary melanoma Breslow thickness and Clark's level with the number of PCR positive markers may be due to the fact that tumor

progression is no longer dependent on these initial pathological parameters of the primary tumor once it has been removed.

EXAMPLE V

Statistical Analysis

To assess the difference between using tyrosinase alone as a marker and using tyrosinase, MUC18, p97 and MAGE-3 together, a coefficient in level for small sample proportion analysis was performed. Assessment of significance of disease stage to PCR data that was analyzed is summarized below:

n = 120	Stage I = 4	NED = 65
	Stage II = 18	AWD = 38
	Stage III = 32	EXP = 17
	Stage IV = 66	

Of the 120 patients, 49 tested negative for tyrosinase. 42 of these tested positive for at least one of the other three markers (MUC18, P97, MAGE-3). This improvement is statistically significant at the 99% confidence level. It can therefore, be concluded that the four marker PCR assay is more sensitive than the single marker (tyrosinase) assay.

Next, an attempt to correlate a patient's disease stage (I, II, III, or IV) and the number of positive markers (0-4) was undertaken. Table 7 shows the breakdown.

Table 7

Number of PCR markers correlated to stage and disease status

	Patients Number of positive markers					
Disease stages	0	1	2	3	4	Total Patients
I	3	0	0	1	0	4
II	2	6	5	5	0	18
III	4	5	15	8	0	32
IV	3	15	23	18	7	66
Total	12	26	43	32	7	120

Positive markers refer to detection of tyrosinase, p97, MUC18 and MAGE-3 by either PCR, nested PCR or Southern blotting.

The results show a positive correlation between stage and the number of positive markers, $p = 0.0025$, *i.e.*, as stage increases, the proportion of positive markers also seems to increase.

In the follow-up period after blood drawing, patients were divided into those with clinical evidence of disease progression and those with no evidence. The number of patients positive for 0 to 4 PCR markers was correlated to disease progression. The relationship between progression and the number of positive markers also was assessed. Analysis showed that there was a significant correlation ($p < 0.05$) between number of positive markers and disease progression. Table 8.

Table 8

Number of PCR markers correlated to progression of disease

	0 pos	1 pos	2 pos	3 pos	4 pos	TOTAL
No Progression	9	13	32	17	2	73
Progression	3	13	11	15	5	47
TOTAL	12	26	43	32	7	120

Thus, although tyrosinase has been used as a marker in a previous report, the studies disclosed herein indicate that tyrosinase alone is not always sensitive in detecting circulating melanoma cells. The use of more than one marker can verify the presence of occult melanoma cells and significantly increase the sensitivity of detecting melanoma cells that express few or no copies of tyrosinase mRNA. The study demonstrated that using four markers was significantly better than tyrosinase alone. In addition, the number of markers detected in individual patients correlated, significantly, with stage and disease progression. This higher expression of individual marker genes indicates, that there is an increase in the heterogeneity of tumor cells or an increase in the number of cells in circulation, at advance stages of disease.

Overall, the level of detection was similar for tyrosinase and p97 markers. MUC18 marker was the most frequently detected whereas, MAGE-3 was the lowest. Although MAGE-3 is expressed in cell lines and biopsies in higher frequency, the number of mRNA copies in a single tumor cell is likely to be very low. This may be related to the state of the cell or clonal phenotype during circulation in the blood.

EXAMPLE VI

Detection of β-HCG mRNA Expression in Melanoma Cells

A. MATERIALS AND METHODS

(i) Melanoma Cell Lines

Twenty-four melanoma cell lines were established and characterized at John Wayne Cancer Institute as previously described (Hoon *et al.*, 1991). Cell lines were cultured and passaged as described in Example I.

(ii) RNA extraction

Total cellular RNA was extracted, isolated and purified using Tri-Reagent according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH) and described in detail in Example I. Cells from melanoma lines were lightly trypsinized and collected from tissue culture flasks. Biopsy specimens if cryopreserved were rapidly thawed and kept in a ice water bath. Tumor biopsies were kept in a ice water bath when being minced. All RNA extraction was carried out in a sterile designated laminar flow hood with RNase free labware. Purified RNA was quantitated and assessed for purity by UV spectrophotometry.

(iii) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. The β-HCG primer sequences were as follows: 5' primer was 5'-ATGCCACCCTGGC TGTGGAGAA-3' (SEQ ID NO: 13) and the 3' primer was 5'-GGGAGTCGGGATGGACTTGGAA-3' (SEQ ID NO: 14). The RT-PCR cDNA product was 367 bp. The 5'primer has only one mismatch with the β-luteinizing hormone (LH, see below)

while the 3' primer is homologous to both β-HCG AND β-LH coding regions. A full-length, PCR product, amplified from β-HCG DNA, was used a probe for Southern blot analysis.

The sequences of α-HCG primers were derived from the GenBank; 5'-AAAGGAGCGCCATGGATTAC-3'(SEQ ID NO: 15); and 3' primer, 5'-CCATTACTGTGACCCTGTTA-3'(SEQ ID NO: 16). The α-HCG PCR cDNA product was 297 bp. The primer sequences for β-HCG/LH receptors were 5'primer, 5'-CCCGATGTGCTCCTGAACCAGA-3'(SEQ ID NO: 17); and 3'primer, 5'-GCTGACACCGACAAGGGGCAA-3' (SEQ ID NO: 18). The RT-PCR cDNA product for β-HCG/LH receptors was 496 bp. The β-actin primer sequences were as follows: 5' primer was 5'-GGAGCAATGATCTTGATCTTC-3' (SEQ ID NO: 21) and the 3' primer was 5'-CCTTCCTGGGCATGGAGTCCTG-3' (SEQ ID NO: 22). The RT-PCR product was 201 bp. The tyrosinase and MAGE-3 primers were the same as described in Example I.

(iv) RT-PCR assay

The RT-PCR assay was carried out as previously described (Morisaki *et al.*, 1992, and in Example I). Briefly, reverse transcription was carried out with oligo $(dT)_{15}$ primer and AMV reverse transcriptase with 5 ug of RNA and incubated for 2 hr at 42°C and 99°C for 5 min. The RT-PCR conditions were set up as follows: 95°C for 5 min followed by 95°C for 1 min, 65°C for 1 min, 72°C for 1 min, and 72°C for 10 min for final primer extension sequence and performed in an OmniGene thermocycler (Hybaid, Middlesex, England).

(v) Restriction digestion

β-HCG is a gonadotropin hormone composed of an α- and β-subunit (Giuliano, *et al.*, 1995; Fiddes *et al.*, 1979; Boorstein *et al.*, 1982). The amino acid sequence of α-HCG is essentially indistinguishable from those of the other human gonadotropin hormones, such as follicle-stimulating, luteinizing, and thyroid-stimulating hormones (Fiddes *et al.*, 1979; Pierce *et al.* 1981). However, the β-HCG subunit is different amongst the other hormone subunits except for the β-LH subunit; they share 82% common amino acid sequence (Talmadge *et al.*, 1984). The ß-subunit of HCG to date has been shown to consist of cluster of 6 related genes linked closely to the ß-LH single gene (Bo *et al.*, 1992). Since the β-HCG and β-LH are highly homologous it is not possible to design a primer sequence absolutely specific to β-HCG.

However, the β-HCG PCR cDNA product has a unique *Sty* I restriction site that is not present in the β-LH PCR cDNA product. Digestion of PCR products with this enzyme allows β-HCG to be destinguished from β-LH. RT-PCR cDNA product was incubated with 10X NEBuffer 3 (New England BioLabs, Beverly, MA) and *Sty* I (10 U/ml) (New England Biolabs) and the mixture was incubated overnight at 37°C. The endonuclease digested product mixture was run on a 2% agarose gel and stained with Etbr. β-HCG RT-PCR cDNA product digested with *Sty* I produces a 271 and 96 bp band. If no digestion occurred the reaction was repeated at least twice to confirm.

(vi) Southern blotting

RT-PCR cDNA products run on a 2% agarose gel were denatured and transferred overnight onto nylon membrane (Micron Separations, Inc.) as previously described in Example II. β-HCG cDNA probe was prepared by PCR,

purified and digoxigenin labelled as described in Example II.

B. RESULTS

Assessment of β-HCG expression in cells by molecular techniques has been difficult because of the sequence homologies of both α and β subunits to related hormones. The terminal end of the β-chain subunit chain was chosen as a target for RT-PCR since it had the most significant differences compared to other related hormone β-chain subunits.

Initially 24 established human melanoma cell lines derived from different anatomical sites were assessed to determine if they expressed β-HCG chain. Oligo $dT_{(15)}$ priming was carried out to assess only poly A mRNA of β-HCG. Of the 24 cell lines tested by RT-PCR, 16 of 24 produced a specific cDNA product of the correct size (367 bp) as verified by Etbr gel staining.

β-actin was run on all samples as an internal control to verify RNA yield and efficiency of the RT-PCR assay. Each assay had a negative control consisting of RT-PCR reagents alone without RNA and a positive control for β-HCG. Southern blot analysis of PCR cDNA product with the β-HCG probe showed that three of the cell lines negative by Etbr staining had a specific cDNA band. However, one cell line in which the Etbr staining was questionable showed no specific band on Southern blot analysis. Overall 18 out of 24 cell lines were positive (75%) for β-HCG marker expression.

To further verify β-HCG marker expression, endonuclease restriction digestion with *Sty I* was carried out on the RT-PCR cDNA products. All cDNA products digested, produced two bands, 271 bp and 96 bp as

observed on Etbr gels, indicative of β-HCG marker. These digested products were further verified by Southern blot analysis with β-HCG cDNA probe.

EXAMPLE VII

Detection of β-HCG mRNA Expression in Melanoma Tumor Biopsy Specimens

A. MATERIALS AND METHODS

(i) Melanoma Tumor Biopsy Specimens and Blood Preparation

Melanoma tumor biopsy specimens that were defined by histopathology as malignant melanoma were assessed. Melanoma biopsies were obtained from primary lesions and from multiple anatomical sites of metastatic lesions from different patients. Specimens were immediately frozen or processed as received from the operating room. In this study liquid nitrogen cryopreserved and fresh tumor biopsies from surgery were assessed. On obtaining melanoma biopsies non-melanoma tissue was carefully dissected away from normal tissue under sterile conditions in a laminar flow hood.

PBL were obtained from 25 normal male and female volunteer donors and the buffy coat was collected for RNA isolation as described in Example I. All other techniques including RT-PCR, Southern blotting and restriction enzyme digests were as described in Example VI.

Normal axillary lymph node tissue that was assessed as histopathology negative for tumor was obtained from melanoma and breast cancer patients undergoing elective surgery. Axillary lymph nodes were assessed by

histopathology for malignancy by standard conventional hematoxylin and eosin (H & E) staining.

B. RESULTS

Melanoma primaries and metastases have been shown to contain infiltrating immune cells (Cochran and Hoon, 1987). To be certain that β-HCG mRNA was being expressed by the tumor cells and not a product of infiltrating lymphoid cells, peripheral blood lymphocytes were also analysed for expression of β-HCG mRNA. PBL from 25 normal volunteer donors were analysed by RT-PCR but no evidence of β-HCG expression was observed, even after Southern blot analysis (except in one individual who was positive in a second blood draw).

Five lymph nodes from two breast and melanoma patients, were found to be negative by H & E staining and by RT-PCR and Southern blotting.

Both cryopreserved and fresh biopsy tissue were anaylsed by histopathology and by RT-PCR, restriction digestion and Southern blotting. Out of 40 patients, 38 were identified, as melanoma positive by histopathology, while 16 were identified as positive by RT-PCR for β-HCG marker. In other words, an estimated 42% of melanoma biopsies were β-HCG positive. All specimens that were found to be melanoma negative by histopathology, were also negative for β-HCG marker expression. The detection of β-HCG mRNA was much weaker in melanoma biopsy tissues as compared to melanoma cell lines. This detected lower gene activity may be due to the heterogeneity of tumors, variability of host physiologic regulation of β-HCG, or simply the dilution of RNA by normal cell infiltrate.

There was no significant difference in β-HCG mRNA detection between cryopreserved and fresh biopsy

specimens. β-actin expression was detected in all specimens, thus verifying the integrity of the mRNA and the PCR assay. α-HCG subunit expression in five β-HCG positive melanoma cell lines and five melanoma biopsies was also analyzed. However, no α-HCG expression was detected by RT-PCR even when followed by Southern blot analysis.

EXAMPLE VIII

A Comparison of β-HCG mRNA Expression with Other Melanoma Markers

A. MATERIALS AND METHODS

(i) Surgical Specimens

Axillary lymph node tissue was taken after elective surgery of TDLN from seven melanoma patients. TDLN were assessed by histopathology for malignancy by standard conventional hematoxylin and eosin (H & E) staining. β-HCG mRNA expression was compared with tyrosinase and MAGE-3 mRNA expression by RT-PCR. All other materials and methods were as described in Example VI.

B. RESULTS

Out of eight tumor-draining lymph nodes (TDLN) (from seven melanoma patients) five were positive for β-HCG expression, six for tyrosinase and three for MAGE-3. In two patients, none of the markers were detected.

Table 9

Analysis of β-HCG expression in melanoma TDLN

TDLN	Path	β-actin	β-HCG EtBr	β-HCG S. Blot	β-HCG Sty I	Tyr	MAGE3
1	+	+	-	+	+	+	-
2	+	+	-	+	+	+	-
3	+	+	-	-		+	+
4	+	+	+	+	+	+	+
5	+	+	-	-		-	-
6	+	+	+	+	+	+	+
7a	+	+	+	+	+	+	-
7b	-	+	-	-		-	-

TDLN refer to individual patient nodes examined (a and b refer to two separate nodes). Pathology refers to the hematoxylin and eosin staining histopathology analysis of lymph node sections. + refers to presence of melanoma metastases and - refers to no metastases. RT-PCR analysis detected by Etbr and Southern blot is indicated as + or -. Tyr refers to RT-PCR analysis by tyrosinase primers followed by nested tyrosinase RT-PCR if negative.

In conclusion, β-HCG is a useful addition to the group of melanoma markers described in Examples I through V. The frequency of expression of β-HCG mRNA in melanoma appears to be similar to that of the melanoma tumor antigens MAGE-3 and MAGE-1.

EXAMPLE IX

Detection of GalNAc mRNA Expression in Melanoma Cells and Biopsies

A. MATERIALS AND METHODS

(i) Melanoma cell lines and Surgical Specimens

Melanoma cell lines were all established at JWCI and grown as described in Example I. 20 melanoma tumor

biopsy specimens were obtained as described in Example VII. RNA extraction and RT-PCR assay was as described in Example BI.

(iv) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. The GalNAc primers used were: 5'-CCAACTCAACAGGCAACTAC-3' (SEQ ID NO: 19) and 3' GATCATAACGGAGGAAGGTC-3' (SEQ ID NO: 20). cDNA probes, amplified by PCR with these primers, were used in Southern blotting, which was performed as described in Example II. The tyrosinase and MAGE-3 primers were the same as described in Example I and β-HCG primers were the same as described in Example VI.

Table 10

GalNAc expression in melanoma biopsies and cell lines

SPECIMENS	GalNAc EXPRESSION		
BIOPSIES# METASTASES			
19	-		
25	-		
68	-		
100	+		
102	+		
178	-		
221	-		
224	-		
246	+		
250	-		
260	-		
261	-		
287	-		
292	+		
295	+		
301	+		
351	+		
361	+		
380	+		
443	+		
MELANOMA CELL LINES			
MATT	+		
M101	+		
M12	+		
M24	+		
M10	+		
M18	+		
MKN	+		

Table 10 (continued)

SPECIMENS	GalNAc EXPRESSION		
MHL	+		
MCE	+		
MKE	+		
MELL	+		
MMAC	+		
MF	-		
M18	+		
PERIPHERAL BLOOD LYMPOCYTES			
DONOR #1	-		
DONOR #10	-		
DONOR #12	-		
DONOR #13	-		
DONOR #322	-		
DONOR #323	-		
DONOR #324	-		
DONOR #325	-		
DONOR #326	-		
DONOR #338	-		
DONOR #339	-		
DONOR #340	-		
DONOR #342	-		
DONOR #343	-		
NORMAL LYMPH NODE #349	-		
NORMAL LYMPH NODE #364	-		

B. RESULTS

As shown in Table 7, detection of GalNAC mRNA was succesfully detected in 13 out of 14 melanoma cell lines and 10 out of 20 biopsy specimens. Furthermore, no GalNAc marker expression was observed in normal lymph nodes or PBL. These are similar results to those found for β-HCG and MAGE-3 in previous examples. Indicating that GalNAc mRNA expression is another marker which may be utilized for the detection of melanoma and metastases.

Amplification of GalNAc mRNA is an indicator of gangliosides, GM2 and GD2, expression. Direct detection of GM2 and GD2 in occult metastases and small tumor lesions such as melanoma primaries is very difficult and often impractical when using standard biochemical methods. Monoclonal antibodies to gangliosides are available but often cross-react with other carbohydrate structures and therefore are not reliable and do not represent absolute ganglioside expression (Hoon *et al.*, 1993).

Detection of tumor cells with the marker GalNAc by RT-PCR provides a novel approach to detect metastatic melanoma and breast cancer cells in blood or fluids that would not be possible by current biochemical or immunological techniques.

EXAMPLE X

Detection of β-HCG mRNA Expression in Breast Cancer Cells

A. MATERIALS AND METHODS

(i) Breast cell lines

The established breast cancer cell line JWCI BM-1 was developed from a primary invasive ductal carcinoma

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patients were positive for all four markers. All six patients were stage IV.

Table 5

Analysis of melanoma patients using multiple marker PCR assay

	Number of patients positive			
Assays	p97	tyrosinase	MAGE-3	MUC18
PCR	16	2	12	80
Nested PCR	-	57	-	87
cDNA blot	49	12	-	-
Total patients	65	71	12	87

Melanoma patients (120) were evaluated. PCR and cDNA blot refer to assays positive for individual marker genes. Nested PCR refer to specimens tested negative for tyrosinase PCR, and + or - for MUC18 PCR that become positive after nested PCR. cDNA blot refers to patients tested that were negative for either PCR or nested PCR and became positive after cDNA blotting.

The PCR analyses were correlated with disease stage and status (AWD & EXP, NED) of patients. The follow-up time for clinical status after blood drawing for PCR analysis was 8-15 months. In the study, there were 4, 18, 32, and 66 Stage I, II, III, and IV patients, respectively. The majority of the patients in individual Stages II to IV were PCR positive. Table 6.

tumor and characterized as a breast cancer line at the John Wayne Cancer Institute. Breast cell lines MDA-MB-231, MCF-7, BT-549, T-47D and BT-20 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured according to instructions provided. The 734B line is an established subclone of MCF-7. Cells were grown in 10% fetal calf serum (heat-inactivated) RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus penicillin and Streptomycin (GIBCO, Long Island, NY) in T75 cm^2 flasks. Adherent cell lines were routinely passaged by trypsinization every 3-4 days. When cell lines attained 75-85% confluency they were used for PCR analysis.

(ii) RNA preparation

Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) was used to isolate total RNA from the cell lines and surgical specimens, following the manufacturer's instructions and described in Example I. One μg of total RNA was used in the PCR assay to detect β-HCG mRNA. Oligonucleotide primers and probes were as described in Example VI.

(iii) RT-PCR

Reverse transcription was as described in Example I, using oligo $(dT)_{15}$ and oligo nucleotides as described in Example VI.

The PCR mixture was also as described in Example I and incubated in an OmniGene temperature cycler (Hybaid, Middlesex, England) at 95°C for 3 min for 1 cycle; 95° C for 1 min, 65°C for 1 min, 72°C for 1 min for 30 cycles; and 72°C for 10 min. The PCR cDNA products were assessed in a 2% agarose gel containing ethidium bromide. A 100 bp DNA ladder (GIBCO BRL Life Technologies Inc.,

Gaithersburg, MD) was used as a bp reference marker. Restriction enzyme digestion and Southern blot analysis were performed as previously described in Example VI.

(iv) β-HCG protein expression in breast cancer cells

Breast cancer cells from individual cell lines were seeded in 12 well tissue culture microplates at 2 million cells/well in 1.5 ml of RPMI 1640 without fetal calf serum and cultured at 37°C in a tissue culture incubator for 24 hr. Supernatant was harvested and concentrated 10 times to a volume of 150 µl using Centricon 10 concentrators (Amicon Division, W.R. Grace & Co., Beverly, MA). β-HCG in the supernatant was measured using a total β-HCG Quantitative Test kit (Medix Biotech Inc., Foster City, CA), following the manufacturer's instructions. Supernatant samples were tested in duplicate with a standard reference for each assay. The ELISA reaction was read at 490 nm using a Vmax kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA).

B. RESULTS

(i) β-HCG mRNA expression in breast cell lines

All seven breast cancer cell lines were found to express β-HCG mRNA. A positive result was indicated by a 367 bp cDNA band as detected by Etbr-staining and Southern blot analysis. To confirm the identity of the amplified PCR cDNA product, samples were digested with endonuclease *Sty I*. All seven β-HCG PCR products were cleaved by *Sty I* to produce bands of 271 and 96 bp on Etbr gels, thus confirming β-HCG mRNA expression. As a negative control, PBL from 25 normal (male and female) volunteers were examined. None of the control specimens

were shown to be positive for β-HCG marker expression by PCR and Southern blotting.

The expression of α-HCG mRNA was also examined by PCR in breast cell lines. In cell lines MDA-MB-231, JWCI BM-1, and T-47D, α-HCG mRNA expression was detected by Etbr-stained gel electrophoresis. The α-subunit detected could be HCG or other related hormones since they all share a high degree of similarity in α-subunit. For this reason α-HCG as a cancer marker, is not practical.

(ii) β-HCG protein expression in breast cancer cell lines

Out of the seven breast cell lines expressing β-HCG mRNA, only three cell lines (MDA-MB-231, T47-D, JWCI BM-1) secreted detectable levels of β-HCG protein as analysed by ELISA (0.15 mIU, 0.15 mIU, 0.1 mIU/2 x 10^6 cells, respectively). The cell lines, producing β-HCG were those that were positive for α-HCG mRNA expression.

Breast cancer lines were also analysed for β-HCG receptor mRNA. Human β-HCG/LH receptor genes cloned recently, have been shown to possess a high dgree of similarity (Minegish *et al.*, 1990). All cell lines were positive for β-HCG/LH receptor mRNA expression. To evaluate whether β-HCG/LH receptor could be used as a marker for metastatic breast cancers, PBL from six normal volunteer donors (male and female) was analysed for the corresponding mRNA using the primers as described in Example VI. All donors expressed β-HCG/LH receptor mRNA indicating that the β-HCG/LH receptor is not a reliable marker for detecting breast cancer cells in blood or lymph nodes.

EXAMPLE XI

Sensitivity of β-HCG Marker

A. MATERIALS AND METHODS

(i) PCR detection sensitivity

The sensitivity of the PCR assay to detect β-HCG mRNA in breast cancer cells was assessed by the following methods:

(a) RNA was isolated from MDA-MB-231 cells and serially diluted from 1 to 10^{-6} μg, and then analysed by the RT-PCR assay.

(b) Cell suspension of MDA-MB-231 cells were prepared and diluted with PBL to produce an *in vitro* model occult carcinoma cells in lymph nodes. 10^7 PBL were mixed with a variable number of cancer cells ranging from 1 to 10^5. Total RNA was then isolated from the mixtures and analysed by the RT-PCR assay and observed on an Etbr gel along side a positive (10^6 MDA-MB-231 cells) and negative (10^7 PBL) control. All other method were as described in Example X. The PBL were obtained from normal volunteers in which PCR analysis had shown no presence of β-HCG mRNA.

B. RESULTS

RT-PCR results of a series of diluted RNA isolated from MDA-MB-231 was determined on Etbr agarose gels. β-HCG marker was detected from as little as 10^{-5} ug RNA. This detection was enhanced ten fold by Southern blotting, enabling β-HCG mRNA expression detection from as little as 10^{-6} μg of RNA.

Employing the *in vitro* model it was shown that one breast cancer cell, determined by the amplification of β-HCG marker, could be detected up to among 10^7 PBL.

EXAMPLE XII

Detection of β-HCG mRNA Expression in Breast Cancer Specimens

A. MATERIALS AND METHODS

(i) Surgical Specimens

Thirty-one lymph nodes were collected from 18 patients (13 invasive ductal carcinoma, 4 invasive lobular carcinoma, and one *in situ* carcinoma) who were undergoing mastectomy with axillary lymphadenectomy for clinically early stage breast cancer. Nodes that were only logistically practical for cutting, for conventional pathological diagnosis and for archive fixation were obtained for RT-PCR. Patients ranged in age from 37 to 73 years old. In order to compare the results of PCR with histological analysis, the lymph nodes were divided into two, one half was analyzed by PCR and the other by histopathological H & E staining of serial sections.

For a negative control, blood was obtained from 25 normal volunteer donors (both male and female). All further materials and methods were as described in previous examples.

B. RESULTS

Table 11 shows the results of β-HCG mRNA expression in TDLN. Two TDLN from eleven patients (patient G to Q) and three TDLN from one patient were analysed. Five of the TDLNs were found to be negative by conventional H & E

staining, were found to be positive for β-HCG marker expression in the PCR assay (No. 4, 16, 19, 23, and 24). If the RT-PCR assays were found to be negative, Southern blot analysis was subsequently performed.

Overall, 9 out of 31 TDLN were found to be negative by H & E staining and PCR with or without Southern blotting. Four of the TDLN which were found to be negative by both PCR and histological examination, were subsequently found to be positive following Southern blot analysis (No. 12, 13, 15, and 22). All 367 bp β-HCG PCR cDNA products detected by PCR or Southern blotting were digested by restriction enzyme *Sty* I. There were no TDLN found to be positive by histological examination, but found negative by PCR or PCR and by Southern blotting.

Table 11

Detection for β-HCG expressing tumor cells in breast TDLN

Specimen number	Patient	Tumor histology	Pathology		PCR	Southern blot
1	A	lobular	+	+		
2	B	ductal	+	+		
3	C	ductal	+	+		
4	D	insitu	-	+		
5	E	lobular	+	+		
6	F	ductal	+	+		
7	G	ductal	-	-		-
8			-	-		-
9	H	ductal	-	-		-
10			+		+	
11	I	ductal	-	-		-
12			-	-		+
13	J	ductal	-	-		+
14			-	-		-
15	K	ductal	-	-		+
16			-	-		+
17	L	ductal	-	-		-
18			+	+		+
19	M	ductal	-	+		
20			+	+		
21	N	ductal	-	-		-
22			-	-		+
23	O	ductal	-	+		
24			-	+		
25	P	ductal	+	+		
26			+	+		
27	Q	ductal	-	-		-
28			-	-		-
29	R	lobular	+	+		

Table 11 (continued)

Specimen number	Patient	Tumor histology	Pathology	PCR	Southern blot
30			+	+	
31			+	+	

Individual patients are labelled as A - R and numbers refer to individual TDLN. Tumor histology represents H & E staining diagnosed pathology. Pathology refers to diagnoses of + or - for breast cancer metastases. PCR results are indicated as + or - on Etbr gel electrophoresis analysis. Specimens negative for RT-PCR assay were subsequently Southern blotted with β-HCG cDNA probe. Southern blot analysis is indicated as + or -.

EXAMPLE XIII

Detection of Breast Cancer Cells by Multiple Markers

A. MATERIALS AND METHODS

(i) Breast cell lines and Surgical Specimens

The breast cell line MDA-MB-231, MCF-7, BT-549, T-47D and BT-20 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured according to instructions provided. The 734B line is an established subclone of MCF-7. Cells were grown as described in Example X. 11 breast cancer biopsies were extracted as described in Example XII. RNA extraction and RT-PCR assay were as described in Example X.

(ii) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and purified at the Molecular Biology Institute Core Facility, UCLA. The MAGE-1 primer sequences were as follows: 5' primer was 5'-GCTGGAACCCTCACTGGGTTGCC-3' (SEQ ID NO: 23) and the 3' primer was

5'-CGGCCGAAGGAACCTGACCCAG-3'(SEQ ID NO: 24). The RT-PCR cDNA product was 421 bp. The tyrosinase and MAGE-3 primers were the same as described in Example I, β-HCG primers were the same as described in Example VI and GalNAc primers were the same as described in Example IX. cDNA probes, amplified by PCR with these primers, were used in Southern blotting, which was performed as described in Example II.

B. RESULTS

Multiple markers were used to analyse breast cancer cells and breast cancer biopsy specimens by RT-PCR and Southern blotting. Tables 12 and Table 13 shows the results. All breast cancer cells were positive for at least five, out of the six, markers. For the biopsy specimens, at least one of the markers were detected from all samples. None of the markers alone would have been able to detect cancer cells in all of the specimens. This variation in marker detection reflects the heterogeniety of tumor cells. In conclusion, multiple markers are more sensitive to detection of breast cancer than any one marker.

Table 12

Analysis of Markers in Breast Cancer Cell Lines

	MAGE3	*MAGE 1*	*MUC18*	*p97*	*GalNAc*	*β-HCG*
BT20	+	+	+	--	+	+
BT549	+	--	+	+	+	+
902P	+	+	+	+	+	+
T47D	+	+	+	--	+	+
734B/24	+	+	+	+	+	+
231/45	--	+	+	+	+	+
MCF7	+	+	+	+	+	+

+/-: RT-PCR or Southern Blotting

Table 13

Analysis of Markers in Breast Cancer Biopsies

Biopsy tumor or number	GalNAc	β-HCG	MUC 18	P-97	MAGE-3	MAGE-1
0350T2A122794	+	+	+	--	--	+
0424T2A011795	--	--	--	--	+	--
0433T2A011895	--	+	+	--	+	--
044T2A012095	--	--	--	--	+	--
0460T2A012695	--	+	+	--	+	+
0498T2K020395	--	--	+	--	--	--
0500T2K020695	--	--	+	+	+	--
0506T2A020795	+	+	+	+	--	+
0520T2A020995	--	--	+	--	--	+
0522T2A020995	--	--	+	--	--	--
0525T2A020995	--	--	+	+	--	--

+/-: RT-PCR and Southern Blotting

Previous PCR studies have not analyzed large numbers of patients with different clinical stages of melanoma or breast cancer. This is important in evaluating the sensitivity and clinical significance of the assay. Furthermore, this information is useful in staging disease into clinical subgroups, in particular, identifying subgroups of patients that need more intensive therapeutic intervention. For example, in NED patients with circulating tumor cells, immediate therapeutic intervention may be a very efficacious means of controlling potential tumor progression and, thus, preventing clinical disease. The detection of circulating cancer cells may also prove useful for monitoring a patient's response to operative and adjuvant therapies.

Applying a multiple melanoma marker method to the evaluation of circulating cancer cells also provides information about the tumor's phenotype. Identification of specific tumor-associated antigen(s) permits the rational use of specific immunotherapy protocols such as monoclonal antibodies and cancer vaccine (Hoon *et al.*, 1993). The PCR assay also provides a rapid monitoring system as a follow-up to determine if a specific therapy is effective.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

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(F) POSTAL CODE (ZIP): 90230

(ii) TITLE OF INVENTION: DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

(iii) NUMBER OF SEQUENCES: 24

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: UNKNOWN

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: USSN 08/406,307
(B) FILING DATE: 17-MAR-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAAGCCGGCC CAGGCTCG 18

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGAGTCCTCA TAGGATTGGC TCC 23

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAAGGCAAC CTCAGCCATG TC 22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTCGACTCCA CAGTCTGGGA CGACT 25

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCATCTTCC GTGTGCGCCA 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTAGCGACCT CCTCAGGCTC CTTAC 25

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTGGCAGATT GTCTGTAGCC 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AGGCATTGTG CATGCTGCTT 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTCTTTATGC AATGGAACGC 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTATCCCAG TAAGTGGACT 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TACCTGGTGG AGAGCGGCCG CCTC 24

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGCGTCTTCC CCATCAGTGT 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ATGCCACCCT GGCTGTGGAG AA 22

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGGAGTCGGG ATGGACTTGG AA 22

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AAAGGAGCGC CATGGATTAC 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCATTACTGT GACCCTGTTA 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCCGATGTGC TCCTGAACCA GA 22

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCTGACACCG ACAAGGGGCA A 21

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCAACTCAAC AGGCAACTAC 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GATCATAACG GAGGAAGGTC 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGAGCAATGA TCTTGATCTT C 21

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CCTTCCTGGG CATGGAGTCC TG 22

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCTGGAACCC TCACTGGGTT GCC 23

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGGCCGAAGG AACCTGACCC AG 22

CLAIMS:

1. A method for detecting melanoma or breast cancer cells in a biological sample comprising the step of amplifying at least two nucleic acids in said sample, said nucleic acids being melanoma or breast cancer markers.

Claim 1

2. The method of claim 1, comprising the steps of:

(a) extracting RNA from said sample;

(b) contacting said RNA with a primer pair that hybridize to one of said melanoma or breast cancer marker nucleic acids;

(c) amplifying said melanoma or breast cancer marker nucleic acids to produce an amplification product;

(d) detecting said amplification product; and

(e) repeating steps (b), (c), (d) and (e) with a primer pair that hybridize to at least one of the other said melanoma or breast cancer marker nucleic acids.

3. The method of claim 2, further comprising the step of preparing at least two pairs of primers complementary to regions of said melanoma or breast cancer marker nucleic acids.

4. The method of claim 3, wherein primer pairs for at least three melanoma or breast cancer markers are employed.

5. The method of claim 4, wherein primer pairs for at least four melanoma or breast cancer cell markers are employed.

6. The method of claim 5, wherein primer pairs for at least five melanoma or breast cancer markers are employed.

7. The method of claim 6, wherein primer pairs for at least six melanoma or breast cancer cell markers are employed.

8. The method of claim 7, wherein primer pairs for at least seven melanoma or breast cancer cell markers are employed.

9. The method of claim 2, wherein said melanoma or breast cancer cell markers are selected from the group comprising; tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG.

10. The method of claim 2, wherein said amplification is polymerase chain reaction.

11. The method of claim 10, wherein said polymerase chain reaction is nested.

12. The method of claim 2, wherein said RNA is total cellular RNA.

13. The method of claim 12, further comprising the step of converting said RNA to cDNA.

14. The method of claim 1, wherein said sample is comprised of a body tissue or body fluid.

15. The method of claim 14, wherein said body fluid, comprising: peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

16. The method of claim 14, wherein said body tissue, comprising bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy.

17. The method of claim 2, wherein said detecting is by gel electrophoresis.

18. The method of claim 2, wherein said detecting is by chromatography.

19. The method of claim 17, further comprising the step of Southern blotting.

20. The method of claim 1, wherein said sample is of human origin.

21. A kit for use in detecting melanoma or breast cancer cell cells in a biological sample comprising:

(a) pairs of primers for amplifying nucleic acids corresponding to the genes for tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and ß-HCG; and

(b) containers for each of said primers.

22. The kit of claim 21, further comprising enzymes and reagents for the preparation of cDNA's.

23. The kit of claim 21, further comprising enzymes and reagents for radiochemical or chromophoric labeling of nucleic acids.

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 96/03442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 00603 (THE TRUSTEES OF PRINCETON UNIVERSITY) 6 January 1994 see page 10, line 26 - page 11, line 8 ---	1-3, 10-20
X	AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol. 94, no. 4, October 1990, page 507 XP000576592 COX C ET AL: "tumor marker sensitivity single versus multiple markers in patients with breast carcinoma" see abstract 77 ---	1-3, 10-20
X	ISREAL JOURNAL OF MEDICAL SCIENCES, vol. 17, no. 9-10, September 1981, pages 865-8, XP000576568 SULITZEANU D: "markers in breast cancer" see the whole document --- -/--	1-3, 10-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
18 July 1996	29.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 96/03442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGY SERIES, vol. 53, 1990, pages 39-54, XP000576588 MERCER D.: "use of multiple markers to enhance clinical utility" see page 44, paragraph 6 - page 46, paragraph 1 ---	1-3, 10-20
X	GYNECOLOGICAL AND OBSTETRIC INVESTIGATION, vol. 34, no. 2, 1992, pages 65-72, XP002008750 FARGHALY S.: "tumor markers in gynecologic cancers" abstract and page 71 ---	1-3, 10-20
A	THE LANCET, vol. 338, 16 November 1991, pages 1227-9, XP002008751 SMITH B. ET AL: "detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction" see the whole document ---	1,23
A	EP,A,0 520 794 (F. HOFFMANN LA-ROCHE AG) 30 December 1992 see the whole document ---	1,23
A	WO,A,90 09456 (BALAZS) 23 August 1990 see claims 1-13 -----	1-3

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